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PAPAIN

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PAPAIN

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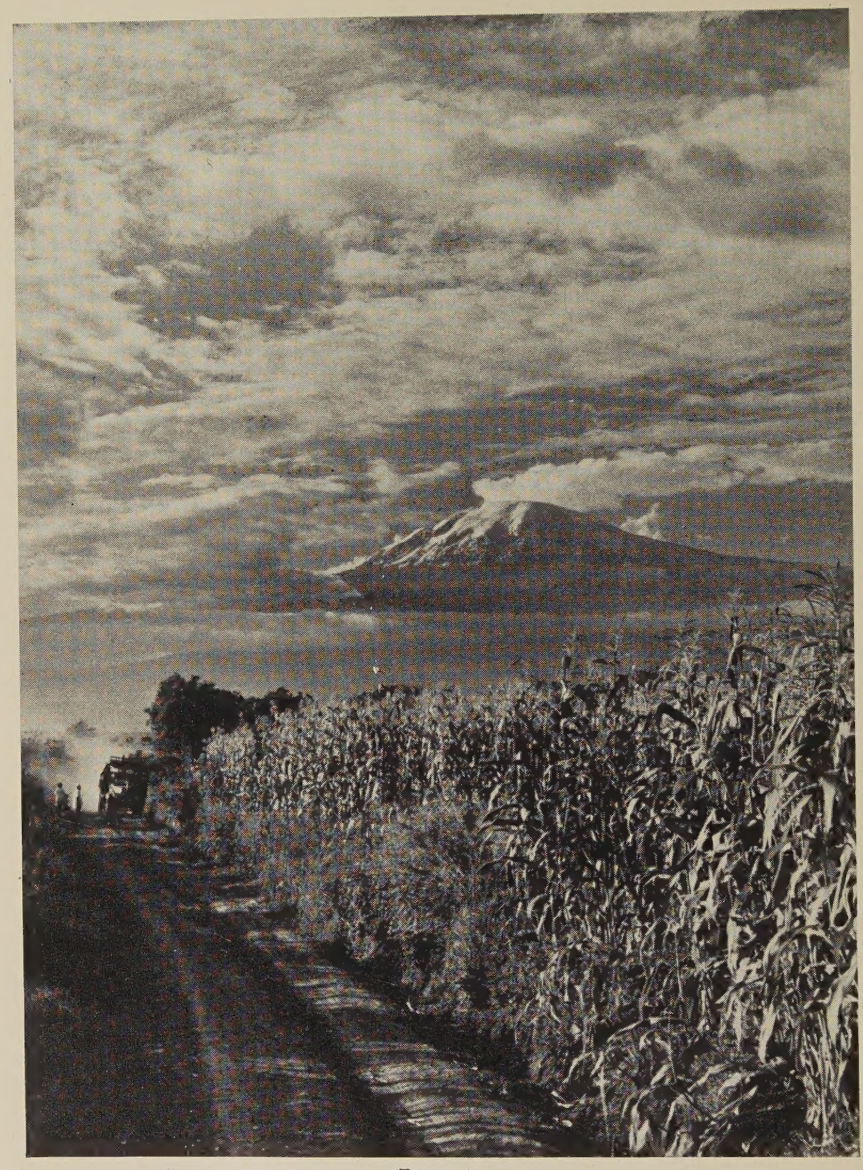

FIGURE 1.

The East African papain industry is centered around Arusha on the slopes of Mount Meru (14,978 feet) and Moshi on the slopes of snow-crested Kilimanjaro (19,565 feet). Above, Kilimanjaro pictured from near Moshi.

FOREWORD

By M. L. Tainter and O. H. Buchanan
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No other plant of the tropics enjoys the popularity and the myriad of uses as does the *Carica papaya*, the tropical papaw or melon tree.

The flowers and fruits of the papaya grow in clusters either just below the crown of the tree or hanging down three or four feet on the stalk-like trunk. The fruits strongly resemble the muskmelon in general characteristics. They are spherical to oblong in shape, usually consist of five longitudinal carpels or sections, and weigh from a few ounces to twenty-five pounds. The skin is thin and smooth in texture, green when immature and yellow to orange when ripe. The yellow to deep orange flesh of the ripe papaya is one to two inches thick and envelops a large, five-angled cavity, attached to the sides of which are membranes containing numerous round, wrinkled black seeds the size of small peas. The *Carica papaya* grows rapidly in its tropical environment and requires only ten to fourteen months from the germination of the seeds to the production of the first ripe fruits.

It is a tall, herbaceous, palm-like tree consisting of a single, slim, erect stalk surmounted by a crowning cluster of large, flat, long-stemmed, palmated leaves from two to four feet in length. The bark is smooth and grayish, marked by large, prominent leaf scars. The trunk is hollow and the wood is soft, white, and spongy. It is native to tropical America, probably having its origin in the West Indies or along the shores of the Gulf of Mexico. During the seventeenth and eighteenth centuries, the plant was apparently taken from the West Indies to Malacca and the Philippines by explorers and traders and thus spread rapidly throughout the tropical islands of the South Pacific. Today, the papaya is found under cultivation in practically all of the tropical countries of the world and in some subtropical regions as far north and south as 32 degrees latitude.

Many uses have been found for the various parts of the papaya tree by the natives of those countries in which it grows. The ripe papaya is the universal breakfast fruit of the tropics, being sliced lengthwise and served much like a cantaloupe. Many enjoy the sweet, resinous flavor without seasoning, while others flavor to taste by the addition of lime or lemon juice, salt, pepper, or sugar. The ripe fruit are also used as ingredients in tropical fruit cocktails, are combined with lettuce and mayonnaise in the form of salads, or are sliced and served as a dessert with sugar and whipped cream. Native papaya tarts, marmalades, sherbets, and papaya glacè are favorite delicacies. The juice of the ripe papaya either is used as a drink or is boiled down to become papaya jelly. The seeds of the papaya are eaten as a delicacy and, when macerated with vinegar, are served as a condiment. The fully grown green fruits are used as vegetables, being boiled, baked, or stewed much like a summer squash, and are also used in the preparation of native pickles. The wood of the

Carica papaya is too soft and porous for use, but the bark is used by the natives in the manufacture of rope.

Native medical lore has credited nearly all parts of the *Carica papaya* with wonderful and mysterious medicinal properties. The large turnip-shaped tap-root possesses an odor of cabbage and a peculiar taste suggestive of radishes. It is used in the formulation of a native nerve tonic. The seeds are regarded highly for their carminative, anthelmintic, and emmenagogic properties and are also used in the preparation of an antipyretic drink for the treatment of various fevers. The sweet, resinous flavor of the ripe fruit makes it an important ingredient in certain syrups, wines, and elixirs which are said to have expectorant, sedative, and tonic properties.

The most important and unique property of the *Carica papaya*, however, is the proteolytic activity of the enzyme papain, which is present in the milky juice or latex of the green fruit and in the leaves and trunk of the tree. Papain is a proteolytic enzyme which in general resembles animal pepsin or trypsin in its digestive action on proteins. A number of the native uses for the green fruit, leaves, and latex depend upon this digestive effect. Both the green fruit and the leaves are used as a bleach for the removal of stains from clothing. The tenderization of tough meat by papayas has long been a common practice in the tropics. The natives rub a slice of the green fruit, rich in juice, over the tough flesh, or dip the meat into a solution of papaya juice for a few minutes before cooking. Slices of the green fruit may also be soaked in the water in which the meat is later boiled. Another practice is to wrap the meat in papaya leaves overnight before cooking.

The fruit is used as a cosmetic, a slice of it being rubbed over the skin to remove extraneous tissue, pimples, and blemishes. The natives make extensive use of the milky juice exuded from scratches in the skin of the green fruit for the treatment of eczema, warts, ulcers, and many types of foul sores, to dissolve the false membranes in the throat in diphtheria, for intestinal worms, and for many other ailments.

The manifold uses for the *Carica papaya* discussed above have resulted in the planting and cultivation of plantations in most of the tropical countries. A large percentage of the papayas grown are consumed locally, although a few are shipped abroad as fresh fruit. In addition, some fruit and considerable quantities of papaya juice are canned and exported to all parts of the world. The main item of commercial importance, however, is the dried latex or commercial papain which is collected and exported for use in various industrial and medicinal fields of application.

Papain is one of the most powerful of the plant proteolytic enzymes, and it is found in all parts of the papaya plant except the roots. The collection and preparation of the latex is a simple process. Usually, only the green fruits are tapped, although papain may be obtained from other parts of the tree. Large mature green fruits are selected, and four or five shallow, lengthwise incisions, not over one-eighth inch deep, are made in each fruit. The flow of latex is then scraped from the fruit. This lancing may be repeated several times at intervals of three to four days. It leaves the fruits badly scarred and unfit for the fresh fruit market, but the quality is not

impaired and the fruits can be used for making juice. The greatest yield of latex is obtained from fruits on a vigorously growing tree during warm weather, early in the morning after a rain. The yield of coagulated latex at one bleeding usually amounts to about 0.1 per cent of the fresh weight of the fruit.

The coagulated latex is dried as quickly as possible to prevent decomposition and a resultant loss of enzymatic activity. Most commercial latex is sun dried, but artificial means, such as are furnished by a fruit drier or one made expressly for the purpose, are preferred. The drying must be carried out at low temperatures, as the enzyme is destroyed by excessive heat. The dry flaky material is usually ground and stored in air-tight containers, since exposure to air inactivates the enzyme. This dried latex or commercial papain is then exported to the United States or to Europe, where it is further processed and sold under various trade names as Caroid,® "papayotin," "papain," *etc.*

Little accurate information is available as to yields. Some collectors figure upon a yearly production of one pound of dried latex per tree, but this is probably a high estimate. The wet coagulated latex reduces down to about twenty-five per cent of its weight in dried powder, which still contains from six to ten per cent moisture. About one-sixth of the powder is probably papain.

The consumption of commercial papain has grown to considerable proportions in recent years, and hundreds of thousands of pounds of the dried papaya latex are now exported from the tropics each year. For years, the United States has been the largest consumer of crude papain, drawing heavily from Ceylon and British East Africa, the two chief sources of supply. In 1941, 251,609 pounds were imported from Ceylon and 49,006 pounds from British East Africa. Four years later, during the war, our imports from Ceylon had dropped to 66,612 pounds, offset by an increase to 232,824 pounds from British East Africa. During 1949, we imported 103,092 pounds from Ceylon, 366,821 pounds from British East Africa, and 5,602 pounds from Belgian Congo, a new source of supply. In addition to these three countries, Siam, the United Kingdom, India, the Union of South Africa, Madagascar, and Cuba have from time to time supplied crude papain to the United States.

Papain is admitted duty-free to the United States with no restrictions placed on its importation. The monetary value of papayas, papaya juice, and papain imported into the United States is estimated to run between one and two million dollars each year. Papayas have been cultivated at experimental stations in Florida, Texas, California, and Hawaii under the auspices of the United States Department of Agriculture, but the high cost of land and labor makes it economically unsound to produce papain in the United States.

The largest quantities of papain are used in the industrial fields: in the manufacture of preparations for tenderizing meats, in the treatment of beer to prevent oxidation and chill hazes, in the manufacture of chewing gum, in the textile industry to prevent wool shrinkage and scratching, as well as to

de-gum silk, and in the tanning industry to bate skins and hides. Also well known, though not so important in the quantities required, are the applications of the enzymatic properties of papain in medicine. Caroid® (one brand of papain) is used widely by physicians for its rapid solvent effect on mucus and its ability to digest sloughing and necrotic tissues. Because of this, it is valuable in dissolving the eschar of burns and the membranes of diphtheria, in loosening the exudates in membranous tracheo-bronchitis, and in gastric catarrh.

Papain is a constituent of antacid powders and other medicaments currently prescribed by doctors for the relief of dyspepsia and other states of impaired digestion or assimilation. Its stability and activity over a wide pH range make it superior to animal enzymes for many purposes in pharmaceutical formulae.

For a product as widely used as papain, the published literature is singularly sparse. Modern studies of the digestive power of the enzyme are not numerous, and even the methods of assaying its potency contain equivocal points which require clarification. Recognition of the need for more definitive studies on this intriguing enzyme led the Sterling-Winthrop Research Institute to send one of its staff into the field to study optimum conditions of collecting and drying the latex and possible means of stabilizing the potency of the resultant powdered enzyme. These studies led to extended consideration of available methods of assay, and finally to study of the digestive activity *in vitro* as well as *in vivo*. Near the conclusion of these studies, we were fortunate in being able to secure from Dr. A. C. Ivy, of the University of Illinois, a critical review of literature on papain which he had prepared for another purpose.

It was hoped that presenting these studies together in a single monograph might enhance their accessibility and usefulness as compared to scattering them through several scientific periodicals. The cooperation of The New York Academy of Sciences, which made this publication possible, is therefore especially appreciated by the authors of the papers which follow.

All photographs supplied through the courtesy of British Information Services, New York, N. Y.

FIGURE 2.

A general view of a papaw plantation in the Arusha district of Tanganyika. The trees, which are in their third year, are spaced nine feet by nine feet to allow for two-way, inter-row cultivation.

FIGURE 3.

On most East African estates, cultivation is done mechanically. Above, a Farmall tractor hauling a 5½ foot disc harrow.

FIGURE 4.

The papaw is normally dioecseous (that is, having male & female flowers on trees). While a certain number of males is necessary for cross pollination—1:50 is the accepted ratio—too many are now favored, as they take up space that could be filled more profitably by fruit-bearing females. Above, a young male plant.

FIGURE 5.

Juma Drefu, African foreman at Sharok Estate Ltd., Usa River, near Arusha, demonstrates the papaw fruit. The skin is green, the pulpy flesh yellow to orange, and the seeds greyish or black.

FIGURE 6.

Tapping in progress. The skin of the fruit is lightly scored and the papain-containing latex drips onto the collecting trays attached to the tree.

FIGURE 7.

A close-up of papain tapping showing the collecting tray (already covered with papain obtained from other trees) in position, and the African tapper making an incision in the skin of a fruit. The large fruit is much scarred from previous tapping and is almost ready for pruning off.

FIGURE 8.

A close-up of a papaw fruit being tapped, showing the cutter used for making the incisions and small globules of latex dripping off the fruit.

FIGURE 9.

The cleaned latex is placed on cloth-covered wire trays and spread out thinly ready for drying in the kiln. To avoid pasting the papain on the cloth a wooden fork is used for the spreading operation.

FIGURE 10.
A typical papain drying kiln.

A REVIEW OF THE LITERATURE ON THE POTENTIAL THERAPEUTIC SIGNIFICANCE OF PAPAIN

By Kao Hwang and A. C. Ivy
University of Illinois, Chicago, Illinois

The digestant action of the juice from papaya, a fruit of the melon tree, *Carica papaya*, Linn., has been known for centuries.[1] The results of the experimental investigation of its action first appeared in 1874.[2] In 1878, Wittmack in Germany also reported the digestive properties of the milky juice of the papaya.[3] When he saw these reports in 1879, Peckolt re-examined a precipitate he had made years before from the fresh juice of the unripe papaya in Brazil and confirmed the digestion of meat in a solution of that precipitate, "papayotin."[4] Later in the same year, Wurtz and Bouchut[5] also published their results of experiments on a purified product from the sap of papaya, designated as "papain." These well-controlled experiments have been referred to as the first scientific investigation of the enzyme.[6] Since that time, the term papain has referred either to the crude dried powdered whole latex from the green papaya[7, 8] or to the same material after purification to various degrees by different methods. Other names, such as papaotin, Caroid, and papoid, have also been used for similar products by different manufacturers. The inadequacy of using the term papain to denote only the proteinase component of the whole preparation[9] is made apparent by the thorough investigation of the complicated enzyme system in this preparation during recent years. The term papain used in this review, unless otherwise specified, will be restricted to designate the whole dried product from the latex from the unripe fruit of *Carica papaya*, which contains proteolytic enzymes and other possible components.

I. *Source and Preparation of Papain*

The plant, *Carica papaya*, is said to have originated in Central America[10] or the West Indies.[11] The most abundant growth of this tree lies between the isothermal lines 77° wherever soil and rainfall are favorable.[10] It is grown by cultivation north and south of these lines (between lines 70°),[10] largely in Ceylon, the Hawaiian Islands, Southern Florida, tropical America, and other tropical countries.[11] It is planted primarily for the production of ripe fruit, which is an important article of food, and secondly for the purpose of producing papain.[11]

Carica papaya is a tree with a single slim straight stalk, about 10 to 30 feet in height in the cultivated plants and up to 60 to 100 feet in the wild varieties.[10] Leaves are large and palm-lobed, with rather deep indentations, arranged at the top of the stalk in the form of an umbrella. The wood of the tree is soft, white, and spongy. The bark is gray (green at the top), smooth, and tough, and is laid in folds and forming rings at intervals. There are several varieties, differing from one another according to the kind of flowers they bear. The trees bearing pistillate and perfect flowers respectively produce rotund fruits resembling the common field pumpkin,

The review by Drs. K. Hwang and A. C. Ivy was made available for publication in this monograph through the courtesy of Dr. Frederick J. Cullen and the Therapeutic Research Foundation, Inc.

and oblong fruits resembling the ordinary squash. The trees bearing the fragrant staminate flowers never produce fruits.

In a vigorously growing tree, fairly large latex vessels, lying just under the epidermis in every part of the plant, are readily observable with a pocket lens.[10, 11] When any of these vessels are severed, a clear, water-like juice exudes freely and rapidly becomes opaque on exposure to air.[12] This milky fluid will run for a short while but soon coagulates and the flow ceases suddenly. The coagulation of the latex is hastened by contact with water[7] and calcium salts[13] and greatly delayed by various substances, such as fluoride, citrate, oxalate,[13] and glycerine.[4] Because of the presence of the calcium salts and pectic compounds in the latex of the papaya, and because the latex of the papaya will coagulate the juice of certain other plants, it has been suggested that this coagulation is due to the action of pectase present in the latex.[10]

Most of the commercial papain has been made from the unripe fruits of the *Carica papaya*. The usual method of preparing papain consists of collecting the milk in a receptacle after making three or four longitudinal scratches in the skin of the green fruit while it still hangs on the tree. The coagulated juice is then spread out thinly and dried rapidly in the air or in a vacuum oven. The dried latex is subsequently powdered and sieved and in this form appears on the market as papain.[14] It is a white or cream-colored powder with a characteristic pungent odor.[15, 16] The scratching process may be repeated several times, as long as the fruit is green.[8, 7] In order to insure good appearance and quality of the papain, the use of metal knives and metal containers in the process of collecting the latex should be avoided,[12, 15, 16] and vacuum drying is always preferred.[7] Different accounts have been given regarding the yield and quality of papain in relation to the time of collection of the latex at various stages of maturity of the fruit.[17, 18] Recent experiments, however, on the yield of the latex obtained at any one time have shown that the weight of the dried latex obtained is a fairly constant proportion (about 0.10 per cent) of the weight of fruit, irrespective of the quantity of wet latex secured. The latter varies widely, probably due to dilution.[19, 7] As the papaya fruit fully ripens, there is little or no latex, and papain is not obtainable.[17, 20]

Papain thus prepared varies a great deal in its activity and has been said to become completely inactive after storage for several months.[7] A new method of preparing better and much more stable papain has been claimed.[14, 21, 22] The addition of sodium chloride to approximately 10 per cent of the weight of the fresh latex, followed by partial removal of the water in vacuum below 55°C. with agitation, results in the formation of a grayish paste having good activity with considerable stability. Weight for weight, the papain pastes had about half the activity of the best dried latex, although they were made from less than one-fourth the amount of wet latex. This may prove to be a practical and economical method for the papain industry.

Recent work has demonstrated papain activity in the pressed juice from the leaves, stalks, flower stems, and bark of the *Carica papaya*.[19, 7] No such activity was observed in the root,[7, 20] although others have reported

the opposite.[23] Seeds of the fruit yield no product like papain,[23, 24] but in an early report[12] it was found that the pressed juice from the seeds was active in digesting meat. Attempts at preparing papain from the pressed juice of the leaves and stalks of the papaya tree are promising.[7] Perfecting a method along this line will be of great value, because not only the plant as a whole, but also the fruitless male trees will be useful.

Before the purification of papain is discussed, activities of papain, other than proteolytic, will be considered.

II. *Enzymatic Activities of Papain*

Most of the experimental studies on papain have dealt with the complicated proteolytic activities of the preparation. While literature on these will be reviewed in detail, other enzymatic activities manifested by ordinary papain will be discussed presently.

The milk-clotting or rennet-like action of papain was probably first studied by Wittmack[3] and shortly thereafter by Peckolt.[5] It is known to be closely related to the proteolytic constituent, from which it has never been separated since the first effort of this sort was made.[25] It is not affected by heating at 70°C. for 90 minutes, and some action is still retained after heating at 90°C. for 60 minutes. It is thus differentiated from calf-stomach rennin, which is rapidly destroyed at 60°C.[26] The kinetics of milk clotting by papain are also different from rennin and chymotrypsin.[27] It was first reported that there was no difference in the clotting of either fresh cow's milk or boiled milk by papain,[4] but it was found that boiled milk was coagulated much more easily than raw milk.[28]

The milk-clotting factor is inactivated by hydrogen peroxide and other oxidizing agents and is restored by reduction with H_2S, cysteine, and hydrogen cyanide.[27, 29] Irreversible inactivation is produced by iodoacetic acid.[29] It is considered to be closely related to the "proteinase" components of the proteolytic system in papain because other similarities are also present. Both have a high temperature optimum and are activated, rather than inhibited, by phenylhydrazine.[27] Crystalline papain and crystalline chymopapain, the two highly purified enzymes thus far obtained from the papaya latex, have been shown to possess equal milk-clotting power, while the proteolytic action of crystalline chymopapain is only one-half of that of the crystalline papain.[30] The ratio of milk-clotting activity to hemoglobin digestion was reasonably constant for different preparations of crystalline papain and both properties decreased to the same extent by the irreversible oxidative inactivation.[31] Samples of crystalline papain of different initial activity may show the same degree of milk-clotting power after preliminary treatment with cysteine.[32] Separate portions of crystalline papain obtained by fractional recrystallization all showed the same milk-clotting activity as the mother crystalline papain.[31] By electrophoretic study of the crude papain, it was revealed that the protein nitrogen and the active constituent, as determined by the casein formol method, by the hippurylamide method, and by milk clotting, migrated to about the same extent and indicated an isoelectric point above 8.5.[31] Experiments on casein digestion and milk clotting with crude papain also gave parallel results.[27] All the findings

cited above tend to support the conclusion that the milk-clotting power of papain represents its proteolytic activity. Contrary to this, there are also reports stating that no relation exists between the proteolytic and milk-clotting powers of papain[33] and that the milk-clotting activity is more susceptible to oxidative influence.[34] Assay of papain by measuring milk-clotting power[27] has been widely used, however, as a quick, simple, and valuable aid in describing the activity of the enzyme complex present in papain.[7, 14]

Kilmer has noted a feeble lipase action of papain.[10] Sandberg and Brand also found a lipase of considerable activity[35] in a crude papain preparation. The enzyme was insoluble in water and could be purified, the usual protein tests being positive. When its activity was measured against olive oil, its optimum effect was obtained at 35–40°C. and pH 5.8 to 6.2 in acetate buffer. Its activity was increased in alkaline medium by the presence of calcium chloride. A later report[36] states that water extracts from two commercial papain preparations exerted marked hydrolytic action on glyceryl triacetate, and that this action was greatly increased when proteins and possibly other substances were added. Apparently, this finding cannot be interpreted on the basis of the lipase component of the papain. Probably, it is a non-specific action of the preparation. Its possible physiological role has been pointed out by the author.

Marked amylolytic activity was observed[10] in the fresh latex of papaya. Boiled starch paste was thinned, and the blue color was changed to purple in a few minutes on addition of iodine. Little reducing sugar was formed, however. Only a trace of this action was present in the dried latex. Some commercial papain may show pronounced amylolytic action, which is believed to be due to the addition of diastase. Other reports state that there is no amylase associated with papain,[25, 37] and that there is no invertase, oxidase, or peroxidase.[37]

In a recent report,[38] Meyer and his co-workers demonstrated the presence of lysozyme in several crude and purified papain preparations obtained from several different sources. Both mucolytic and bacteriolytic activities were shown by all samples but indicated a very low titre as compared with egg white lysozyme and ficin lysozyme. The lysozyme activities of papain and ficin were not in any way correlated to their milk-clotting and proteolytic power, and the egg white lysozyme preparations, even in high concentration, showed no milk-clotting or proteolytic action. Ficus lysozyme has a pH optimum between pH 3.2 and 4.2, while the optimum for egg white lysozyme is pH 5.3. The optimum pH for papain lysozyme was not reported.

It has been observed[39] that papain dispersed the follicle cells which surround the recently ovulated ova of the rat with a rapidity equal to that seen with hyaluronidase, but papain seemed to damage the ovum at the same time. There has been no specific report, however, concerning the possible presence of hyaluronidase in papain preparations.

III. *Purification of Papain*

Experimental workers have long been interested in the chemical nature and mode of action of the proteolytic enzymes in papain. In order to

achieve purification, various procedures have been employed to reduce the content of non-proteolytic components in the fresh latex or in the commercial preparations.

Careful analysis of the sun-dried latex of *Carica papaya*[10] has shown that it contains moisture (6.06 per cent, including ash) and water soluble substances which are mostly proteins consisting of globulin, albumin, proteases, and peptones. The precipitate from a water extract of the latex obtained by saturation with ammonium sulfate has been shown to contain a fraction with greater enzymatic action. The extractive substances consist of coloring matter, "vegetable extractive matter," hard and soft waxes, hard and soft resins, a volatile resin, a substance of the nature of fatty acids, and pectose compounds. There is a constituent possessing corrosive properties among these extractive substances. Persons who handle the green fruit in the preparation of pickles are troubled with raw and bleeding fingers. This constituent is not volatile and remains in the dried juice. It can be removed by chloroform and ether, and is either removed or destroyed in some of the processes of separating the enzymes (precipitation).

Albumin and its degradation products have long been noticed in the latex.[40] and some recent reports state that almost half of the proteins in the fresh latex have no proteolytic activity.[7, 14] Other impurities present in the latex may be inhibitory,[41] destructive, or augmentative[42–45] to the activity of the enzyme.

In the early studies of papain, Wurtz first purified the papaya latex by precipitating the vacuum-concentrated watery extract with ten volumes of absolute alcohol. The white precipitate was collected on filter paper after 24 hours and dried in vacuum.[5] Later, he tried dialysis to remove the more diffusible ingredients, and the same method of alcohol precipitation, but preceded by preliminary treatment with lead acetate.[40] Peckolt also prepared a white odorless substance from the latex of *Carica papaya* by alcohol precipitation and named the product "papayotin."[4] Methods involving similar principles have been adopted by later investigators in this field.[46–48] About 30 methods of separation of the enzyme from the latex have been tried by Kilmer.[10] His method of choice was to treat the carefully dried latex with ether, followed by chloroform, benzine, and, finally, alcohol. The product was a fine gray-white amorphous powder, almost completely soluble in water and more active than the product obtained by other methods.

The purification technique of Willstätter and his group, using alcohol precipitation, has increased the activity two- to three-fold, as measured by gelatin hydrolysis.[49] Purification by adsorption on alumina and other agents was studied by the same group,[49, 50] and one of the resulting materials was claimed to have a ten-fold increase in activity.[50]

By combination of the methods of adsorption and precipitation, Okumura[51] has claimed a highly purified papain with a 10- to 20-fold increase of its specific activity.

Fractionation by various salts has been employed, and crystalline papain was thus isolated from the wet papaya latex[32, 31] and commercial papain, but not from amorphous material.[32] The activity of this crystalline papain, as measured by milk clotting or casein digestion, was 25 to 50 per cent

higher than that of any of the amorphous laboratory preparations and was about twice as great as that of the best commercial preparations.

The crystalline papain thus isolated represents only a minor part of the total proteolytic activity. Another proteolytic enzyme is present in the latex in considerably greater amount and has also been isolated in crystalline form by a different procedure of fractionation.[30] It is designated as crystalline chymopapain because it has a ratio of milk-clotting power to protein digestion (hemoglobin) exactly twice that of crystalline papain. It should be noted that the activity of these two crystalline enzymes, computed per unit of protein nitrogen, is equal with respect to milk clotting.

While the chemical nature and enzymatic activities of papain will be discussed later in detail, some physical properties of purified materials will be given now.

Both Peckolt's papayotin and Wurtz's papain were white amorphous powders easily soluble in water[4, 5] and glycerin[4] but insoluble in alcohol,[4, 5] ether, chloroform, petroleum ether, and volatile and fatty oils.[4]

According to Balls and Lineweaver,[31] crystalline papain occurs in the form of needles, homogeneous under the microscope. In undiluted suspensions, they have a mat-like appearance similar to that of pepsinogen. Sometimes, another form of crystals, large plates with elongated hexagonal faces, less soluble in water, appeared in suspensions of needles after standing at 8°C. for several months. Needle crystals can be prepared from the hexagonal plates, and both have approximately the same proteolytic activity. Crystalline papain is soluble in water and 70 per cent ethyl or methyl alcohol[31] and remarkably stable in 9 M solution of urea[52] but easily salted out from solution, especially at low temperature.[31] The molecular weight of crystalline papain, as determined by the osmotic pressure method, was found to be 27,000 ± 2,000 and the isoelectric point about pH 9.[31]

The crystalline chymopapain occurs in broad, sabre-like needles and sometimes in obvious plates.[30] It is many times more soluble than crytalline papain at the same salt concentrations and pH.[18]

Whether the sum of the activities of these two crystalline enzymes represents the total proteolytic activity of the fresh latex and in what proportion they exist in the purified amorphous papain obtained by various precipitating processes have not yet been studied. For these and other reasons, such as the marked influence of minute amounts of impurities or the effect of oxidation on the activity of papain,[31] the purity of papain requires special definition. Consequently, comparison of the data of different products can hardly be made.

IV. *Chemistry of the Proteolytic Enzymes in Papain and Their Action*

1. *Chemical Nature and the Active Group.* The proteolytic action of papain was shown early to be associated with the protein constituents of the papaya latex,[25, 10] over half of which is now known to be enzyme protein.[7, 14] Purified proteolytic enzymes, crystalline papain and crystalline chymopapain, have been isolated from papain and both are protein in nature.[30–32, 52] They have the same amount of activity on milk clotting, but the chymo-

papain is less active as a proteolytic enzyme. The data on both crystalline enzymes, regarding their chemical nature, are scanty at present, but crystalline papain is relatively better understood. The total sulfur content of the latter enzyme is 1.2 ± 0.1 per cent[31] which is less than that of papain purified otherwise.[53, 54] Its nitrogen content, 15.5 ± 0.1 per cent,[31] is also slightly different from previous reports.[46, 55] Although tyrosine was found on acid hydrolysis of purified papain, it was not found in the ultraviolet absorption spectrum of the intact papain solution.[56] With improved technique, however, both tyrosine and tryptophane have been shown in the intact molecule of papain and exist in a ratio not less than 4:1.[57] No phosphorus was found in crystalline papain.[31]

The nature of the active group concerned in the proteolytic activity of papain is still controversial. Ever since the discovery that hydrocyanic acid, formerly used as an antiseptic in the study of papain digestion, actually accelerates the proteolytic action of papain,[6] great interest has been aroused in regard to its mechanism. Much light has been thrown on the mechanism of the proteolytic action of papain and the nature of its active group by studies using various activating and inhibiting substances. Conclusions are still discordant, however, apparently because of the instability and the difference in the degree of purity of the enzyme preparations.

The sulfhydryl group has been suggested as the active group in papain,[58] which thus accounts for the reversible inactivation of papain by oxidizing agents. This explanation of papain activity in terms of thiol chemistry has been accepted by various workers.[58–61] Other studies have led to the conclusion that the sulfhydryl group is essential only for the activity of a part of the proteolytic system in papain,[62–67] some other essential group being present for the whole or part of the proteolytic system. The milk-clotting activity is also considered to be associated with labile sulfhydryl groups.[29] Crystalline papain gives a negative nitroprusside test, however, and is not titrable by porphyrindin, unless the enzyme is first denatured.[68] After careful titration with iodoacetic acid, cystine, and iodine, the conclusion was reached that one potential sulfhydryl group of about ten in the papain molecule is essential for its activity.[63] Crystalline chymopapain, on the other hand, gives a strongly positive nitroprusside test without preliminary denaturation or addition of other agents.[30] The opinion has been expressed that this may be due to impurity adsorbed on the enzyme,[34] but no positive evidence has been presented.

Some reports totally deny the presence of —SH or —S—S— groups in papain and consider that the aldehyde group is essential for both "proteinase" and "peptidase" activities of papain.[51, 69, 70] Other studies of papain purified from fresh latex[34, 71] affirmed that some preparations showed strongly positive nitroprusside tests while others were negative, but activities of "gelatinase" and "peptonase" (similar terminology to "proteinase" and "peptidase") were present in all preparations. When the nitroprusside test became negative after treatment with copper salts, the enzyme activities were still retained. The dienol group is considered to be necessary for the activity of the enzyme, while the —SH group in papain only acts as

an indicator of the oxidation and reduction of other essential groups of the enzyme susceptible to oxidation and reduction.

2. *The Extent of Digestion.* Results of earlier workers have been conflicting regarding the end products of papain digestion of protein. The difference in results has been attributed to the different antiseptics used in the prevention of bacterial putrefaction during the course of digestion.[72] This was further elaborated by the study of Mendel and Blood, who definitely established the activating effect of hydrogen cyanide and hydrogen sulfide on papain.[6] Workers using antiseptics of this kind gave positive reports of various amino acids formed as a result of papain digestion of casein, egg albumin, or blood fibrin.[6, 10, 25, 72–74] Otherwise, a long period of digestion was needed and the yield was small.[75, 76]

Opinion now seems unanimous that papain digests proteins to the stage of amino acids under appropriate conditions. Peptides and amino acids were obtained from gelatin by the action of papain purified by adsorption.[49] Certain simple substrates of di- or tri-peptides are hydrolyzed by papain and trypsin but not by pepsin.[77] Using an ultra-centrifuge technique,[78] it was found that during the digestion of crystalline ovalbumin by papain, there was a gradual diminution of the albumin molecule resulting from the splitting off of fractions of low molecular weight. Another report states that one-third of the same substrate was split by pancreatic proteinase. While pepsin caused no further change, papain digested another third. When the crystalline egg albumin was first subjected to the action of papain, the digestion was found to be twice as extensive as that by pancreatic enzyme, and, following the action of papain, there was no further effect by either pepsin or trypsin.[79] About 47 per cent of the total nitrogen of casein was liberated as amino nitrogen after 12 days digestion by papain.[80] This was calculated to be 60 per cent of the peptide bonds, about three times the figure estimated from the results of a similar study using crystalline papain.[52] It is stated that the extent of digestion of casein with crystalline papain is not greatly different from that with crude papain.[52]

Digestion of cattle fibrin for 20 days under carefully controlled conditions resulted in the liberation of all the tyrosine and considerable amounts of the phenylalanine, tryptophane, leucine, and isoleucine present in the protein. When only the "proteinase" fraction of papain was allowed to work, occasioned by the digestion taking place under the inhibitory influence of phenylhydrazine, the resulting tyrosine was one-third of that obtainable by acid hydrolysis or the action of whole papain. It was thus concluded, contrary to some earlier studies,[77, 82] that proteinases are not limited to acting on substrates of high molecular weight.[81] Recent work has shown that leucine is probably chiefly located at the end of the protein molecule chain, which perhaps explains the difference in the digestive products of casein produced by papain and pepsin.[83] The idea that the resistance of protein to enzyme digestion is related to its disulphide linkage[84] is supported by the experiments of digesting powdered hoof protein by various digestive enzymes, including papain.[85]

3. *Papain Activity and Protein Denaturation.* Although it is believed that

denatured proteins are more easily digested enzymatically than are the native substrates, there is no consistent opinion in the literature on papain concerning this point. Studies of the digestion of vegetable albumin by papain revealed that heat-coagulated protein was digested with more difficulty than the noncoagulated.[86, 87] During digestion of beef by papain, the rate of proteolysis was not increased by previous cooking of either ground or unground meat.[88] On the other hand, prolonged treatment of wheat by hot water at 90° made the protein much more susceptible to digestion by papain.[89] Denatured hemoglobin and human serum albumin were also digested much more rapidly than when they were in the native state.[90, 91] The nature of the substrate under study may be an important factor, because it has been shown that egg albumin, pseudoglobulin, and euglobulin, when heat denatured, are digested with greater rapidity by papain than when native, while fibrinogen and myosin are digested at about the same rate by papain before and after denaturation.[92]

Denaturation of protein may occur before the molecule is split into fractions by the proteolytic enzymes. This idea is supported by studies with papain acting on different proteins. By means of the ultracentrifuge, papain digestion of crystalline ovalbumin was studied. It was found that the first change of the native protein was the production of molecules of highly asymmetrical shape without change in molecular weight.[93] In another investigation of papain digestion of the same substrate, no unchanged albumin molecules were found on the basis of studies by ultracentrifuge, diffusion, cataphoresis, and light absorption.[94] Denaturation of hog thyroglobulin was also catalyzed by active papain before hydrolytic fission of the protein took place.[95] In a more recent article,[96] the literature has been reviewed regarding whether denaturation of protein is a necessary step prior to hydrolytic splitting. It was found by ultracentrifugal analysis that beef serum pseudoglobin and diphtherial antitoxin was split by papain, either crystalline or crude, into halves, quarters, and dialyzable fragments, which gave no evidence of denaturation. The validity of this conclusion, however, has been questioned.[97]

V. *Factors Which Influence the Action of Papain*

1. *Temperature.* It is recorded that the natives of the tropics tenderized their meat by boiling it with the unripe fruit of papaya or with its juice.[98] This remarkable phenomenon has been reported by numerous investigators in this field since the early days. Wurtz found that a sample of papain still retained its activity after being heated to 105°C.[40] This is supported by the finding[99] that papain resisted dry heat at 100°C. for three hours. When it was in solution, however, its activity was destroyed by heating for 30 minutes at about 82.5°C. Dry heating of papain in the powder form at 100°C. for one hour was used for its sterilization, and there was no deterioration after such treatment.[100] Other reports indicate that papain activity at boiling temperature is not destroyed in a short period of time[10] or that it is rapidly lost under such conditions.[47] Recent studies on purified papain showed that thermal inactivation of papain in solution took place at 75°–83°

and followed the equation of a simple first order reaction.[101, 102] Loss of activity of crystalline papain was also observed first near 70°C.[52] Probably on account of the stability of papain at high temperature, one method of preparing papain from fresh latex of papaya is to heat it at 70°–90° in an inert gas in order to destroy admixed impurities and then to dry it at about 50° *in vacuo*.[103] This marked thermostability of papain has been compared to the comparative stability of the plant proteins to heat.[6] It is also suggested that the proteins present in the fresh latex of papaya protect papain from destruction by heat, while papain itself is not thermoresistant.[104]

Remarkable activity of papain at low temperature was also noticed,[47] even at 10°C.[10] The early finding that papain deteriorated rapidly on standing with native egg albumin or blood serum at low temperature[105–108] was not confirmed by later workers.[6]

Although most laboratory workers have used the temperature of 40°C. for the experiments on papain digestion, the optimal temperature for this enzyme is probably much higher than this. It was pointed out in an early report[3] that the juice of *Carica papaya* had better activity at 60° to 65°C. Again it was found that papain digested more meat protein at 70° than at any lower temperature.[109] Others reported that papain digested the proteins of egg white and serum so rapidly at high temperature (80° or 90°C.) that, when a slightly acidified mixture of papain and the substrate was quickly heated to boiling, most of the protein material became incoagulable.[105, 106] These observations have been confirmed[107, 108, 110] and further elaborated.[6]

2. *pH of the Medium.* One of the peculiarities of papain is its proteolytic activity over a wide range of pH of the reaction media. Early statements that papain is active in acid, neutral, or alkaline medium[5, 6, 101, 111] received recent confirmation with purified crystalline material.[52] Crystalline papain was found still to possess activity (milk-clotting) after standing at 30°C. for two hours between pH 3 and pH 12. In agreement with an earlier report,[50] the optimum stability of papain solution was also in the range of pH 5 to 6. The enzyme was rapidly inactivated near pH 2 and 13, however. Kinetics of the inactivation are probably complicated, as it is pointed out that the per cent inactivation in the first 15 minutes was unexpectedly large compared to that occurring during the next 105 minutes. There are no data on the stability of crystalline papain in the presence of various activators and impurities. Crystalline chymopapain, on the other hand, seems much more stable in acid. It was found that the proteolytic action of this enzyme was undiminished for several weeks at pH 2 (when the temperature was 10°C.).[30]

The pH optimum for the proteolytic action of papain has been a long-debated subject. The difference in opinion is apparently due to the difference in substrates used by various investigators. When the results based on the same substrate are compared, however, the pH optimum of the proteolytic activity of papain found by most workers, as measured by the rate of the initial digestion of the protein, is more or less consistent.

The optimum pH for the action of papain on gelatin has been claimed to be at 5,[111, 112] 4.8,[113] 4.9, [114] or 5.2,[52] all of which are very close. After

completely oxidizing the SH group of natural papain by H_2O_2 or alloxan, the optimum pH for gelatin hydrolysis by SS-papain was 3.6–3.8, while that for hydrolysis by SH-papain was spread over the range pH 3–5.[66]

The optimum pH for the action of papain on fibrin has been reported as 7,[112, 115] but some others noticed two pH optima, 2.5 and 11.[116–118] The optimal action of papain on casein has been found at pH 7[115] or pH 6.5,[52, 119] but other reactions have been used in the study of casein digestion by papain, namely pH 6.5,[80] pH 4.62[120] and pH 7.5.[121] Maximal digestion of denatured ovalbumin by papain took place at pH 7–7.5[52, 122] or pH 5.7–5.9,[114] while earlier workers chose pH 5 for its digestion.[78, 93, 94] Other pH optima for the action of papain are: pH 5 for peptone,[112] pH 4.9 for serum globulin and pH 5.7–5.9 for serum albumin,[114] and pH 6.5–8.5 for hemoglobin.[122]

For the hydrolysis of the synthetic simple substrates by papain, Bergmann and his co-workers have chosen pH 5 in their studies. Others have found two pH optima, pH 5 and pH 6.8–7, for action of papain on such simple substrates as benzoyl-1-arginamide and carbo-benzoxyisoglutamine.[123] According to a very recent study, there is but one optimum at about pH 5 for such substrates and the same pH is also optimum for the "peptidase" action of papain on the casein split products.[124]

The concept that the pH optimum of proteolysis by papain coincides with the isoelectric point of the substrate, as proposed by Willstätter and his group,[49, 112] has been prevalent among a number of workers. This, however, is considered invalid, since both the pH optima for egg albumin and casein are on the alkaline side of their isoelectric points.[52] In the same study, the author used both crystalline papain and commercial papain, which were shown to have the same pH optimum toward casein (pH 6.5).

In recent experimentation[124] with purified papain, it was found that approximately 50 per cent of the peptide bonds of casein were rapidly hydrolyzed at pH 5, releasing 30 per cent of the amino acids as free amino acids. Under comparable conditions at pH 7, only 25 per cent of the peptide bonds were split, although the latter pH is the optimum for the initial rate of digestion. It is claimed to be the first demonstration that the optimum pH for the initial rate of enzymic digestion of a protein is not that for its complete digestion. However, this finding apparently is in accord with the early report by Willstätter and his co-workers on the pH optima for papain-HCN digestion of fibrin and peptone.[112, 125] For this reason, the proposal that proteinases be described as "acido-," "baso-," or "neutroproteinases," depending upon the optimum pH for their activity,[126] is considered inapplicable to papain.[124]

3. *Effect of Oxidation and Reduction.* Activation of papain by hydrocyanic acid and hydrogen sulfide has been found to be a reversible reaction.[6] This has been confirmed and subjected to further studies by later workers. Various oxidizing agents, such as iodine, bromine, dilute hydrogen peroxide, or atmospheric oxygen, inactivated papain under appropriate conditions, while hydrocyanic acid, hydrogen sulphide, cysteine, or reduced glutathione restored its activity.[58–60, 69, 127–129] These findings have been explained by the thiol nature of the enzyme, being active in the reduced state but inactive

when oxidized[58, 60, 129] This subject has been critically reviewed by Hellermann,[9] and the consensus at that time is represented by the following equation:

$$\underset{\text{(active)}}{2\ \text{En-SH}} \underset{\text{(reduction)}}{\overset{\text{(oxidation)}}{\rightleftharpoons}} \underset{\text{(inactive)}}{\text{En-S-S-En}} + 2\ (\text{H})$$

Many thiol reagents have been shown to affect this reaction, and apparently a stoichiometric relationship is present during the process of activation and inactivation.[58]

In the above studies, however, apparently only the "proteinase" activity of papain was taken into consideration. Other activities of papain characteristic of "peptidase" have been shown to behave differently towards a number of reagents under certain conditions.[63, 130] Later work demonstrated that the discrepancies between the papain proteinase and papain peptidase responses to different reagents in the activation-inactivation reactions were due to some impurities present in the enzyme preparation, and the hypothesis of a two-enzyme system in papain was given up.[131] Upon testing purified papain with simple synthetic substrates, it was found that the precipitate obtained when papain is precipitated from a solution containing hydrocyanic acid by isopropyl alcohol behaves like an unactivated papain.[132] This was considered as not readily explained by the oxidation-reduction theory, but that this inactivation during the precipitation could be caused by air oxidation.[133] In subsequent experiments, however, it was demonstrated that inactivation of papain still took place when the hydrocyanid acid was removed *in vacuo*, which involves no process of oxidation.[134] On the basis of this finding, these authors considered that the previously reported observations on the reversible activation of papain by oxidation-reduction processes were due to their effect on the accompanying impurities (natural inactivators) in the enzyme preparation. In conclusion, these authors believed that papain exists in two inactive forms. The α-form is not activated by HCN, but may be converted by H_2S into the β-form, which is then activated by HCN or H_2S. This activation of the β-form consists of the formation of dissociable compounds of the β-form with the activator. This is rather close to the "kinase" theory for the HCN activation of papain.[112]

As the specificity of papain was found to be similar to that of crystalline pancreatic trypsin,[135] papain has been designated as papain trypsinase.[136, 137] Careful studies on purified papain trypsinase demonstrated that α-papain trypsinase and β-papain trypsinase were mutually reversible by processes of oxidation and reduction but that both were inactive. The eventually active enzyme was a dissociable compound of the trypsinase with the activator.[137] This is shown by the following equation:

$$\underset{\text{(inactive)}}{\alpha\text{-Papain trypsinase}} \underset{\text{Oxidation}}{\overset{\text{Reduction}}{\rightleftharpoons}} \underset{\text{(inactive)}}{\beta\text{-Papain trypsinase}} \underset{\text{Evacuation}}{\overset{H_2S}{\rightleftharpoons}} \underset{\text{(active)}}{H_2S\text{-}\beta\text{-Papain trypsinase}}$$

Under strict anaerobic conditions, additional evidences were obtained that the proteolytic coefficients for papain trypsinase depend upon the nature of the activator employed.[136]

More recently, some critical experiments have been carried out on crystalline ficin, a papain-like enzyme from *Ficus carica*.[138] By means of an efficient anaerobic dialysis, or by evacuation of the activator in an atmosphere of purified nitrogen gas, the removal of the activator HCN or H_2S still left an active enzyme, but, if the dialysis was carried out in the presence of air, the enzyme was (reversibly) inactivated. These authors concluded that ficin and other papain-like enzymes were active in a reduced form and were inactivated by mild oxidation. The apparent coenzyme function of cyanide and other thiol compounds was considered a result of the protection of the enzyme from oxidation by atmospheric oxygen or combination from heavy metals. A new nomenclature of the proteolytic enzymes of the higher plants[139] was suggested on the basis of this work, and its validity has been discussed.[140]

Another theory which has been proposed is that the activation of papain involves a surface phenomenon.[141] The activators exert their effect at the interface between the enzyme and the substrate. This explains the inhibitory effect on the enzyme when the concentration of the activator is high. Because of this and the lack of stoichiometric relation in the process of activation of papain, these authors rather doubted the oxidation-reduction theory.

4. *Effect of Physical Agents.* Regarding the effect on papain of physical agents other than heat, the studies on ultraviolet irradiation should be mentioned. It has been shown that ultraviolet irradiation at first activated papain and cathepsin by its reducing effect, as demonstrated by the formation of cysteine from cystine.[59] The increase in the proteolytic index of skin and blood after ultraviolet irradiation was ascribed to this effect. Further exposure to the ultraviolet light caused the papain solutions to become inactive, however. This inactivation of papain by ultraviolet light has been recently confirmed.[142] It was found that the energy required to inactivate a solution of commercial papain was dependent on the wave length and the intensity of the ultraviolet light and the angle at which it struck the solution. As pointed out by Greenberg and Winnick,[127] further studies with pure enzymes should be made.

5. *Effect of Various Chemicals and Biological Agents.* Ascorbic acid has been well known for its reversible oxidation and reduction. Its effect on papain has been studied, but the results are discordant. It was shown to have no activating effect on papain,[41,59] or its effect was inhibitory.[143–145] It has also been reported as having either an activating or inhibiting effect on papain depending upon the kind of substrate.[146] On the other hand, the observation that ascorbic acid-Fe^{++} complex had an activating effect on papain has been made by several workers.[61, 143–146] Various accounts have been given in explanation of these phenomena, but the presence of impurities, either in the enzyme preparation or in the substrate employed, should be considered. It has been shown[61] that the activation of papain

by the ascorbic acid-Fe^{++} complex did not take place when the purified enzymes were used. Fixed S—S protein or oxidized gluthathione had to be present for the activation to occur.

Many studies have been made of the effect of iodoacetic acid on papain. Opinion is almost that iodoacetic acid causes irreversible inactivation.[29, 62, 66, 68, 69, 122, 133, 147, 148] There are reports, however, in the opposite view.[129, 150] They found that active papain was precipitated by alcohol after complete inactivation by iodoacetic acid. On repetition of the same experiments, however, this conclusion was not confirmed.[122, 133] The mechanism of the inactivation of papain has been differently interpreted. It is claimed that iodoacetic acid inactivates papain by destroying its essential SH group.[147] This was doubted, since the iodoacetic acid inactivation of papain was shown to be an immediate reaction.[128] Furthermore, this inactivation was not prevented by the presence of cysteine, and subsequent treatment with cysteine did not bring back the activity of papain, in spite of a strong nitroprusside reaction.[69, 149] It was also shown that the irreversible inactivation of the enzyme was not accompanied by loss of its SH reaction.[62] Others found that iodoacetic acid inactivated both SH and S—S papain.[66] They are of the opinion that this chemical reacts with an essential group of papain other than thiol.[62, 66] Experiments on crystalline papain have demonstrated that inhibition of papain was produced by molecular equivalents of iodoacetic acid, and HI was detected as a product of the reaction.[68]

The effect of metal ions on papain varies according to the kind of chemical, and the interpretations regarding their mode of action and significance are especially controversial. Zinc has been reported to inactivate the gelatin dissolving action of papain, even in excess of HCN,[150] while others have claimed that its toxicity was annulled by the addition of HCN on account of complex ion formation.[151] Krebs[152, 153] found that a number of metallic ions, such as copper, gold, silver, zinc, cadmium, and mercury, caused inhibition (reversible) of papain even at very low concentrations, while a series of other metals had no effect at all. He believed that such toxic metals may exist as impurities in the protein preparation employed as substrate and therefore exert an inhibitory effect on the enzyme. Activation of the enzyme, therefore, was considered due to removal of such inhibitory substances by HCN or H_2S. Additional evidences were given that citrate or pyrophosphate also activates papain by the same mechanism. While the inhibitory effect of these metals is generally recognized, the theory of the activation by their removal, rather than the direct effect of the activator on the enzyme, has been opposed.

When gelatin was made free from metals, papain activity determined with this substrate, in contrast to Kreb's report, was still much increased by HCN or glutathione.[154] Another report[155] was also opposite to Kreb's conclusion. It was demonstrated that the activation of papain by citrate was not, as maintained by Krebs, interchangeable with cyanide, but that either salt acted additively to the effect of the other. Cyanide activation of papain is not a salt effect as manifested by citrate and acetate, which extend the zone of pH activity of the enzyme. Krebs also reported that

Fe^{++} had no effect on papain, even at high concentration.[152] According to another observer,[41] Fe^{++} increased the activity of papain by removing a natural inhibitor. When papain was freed from this impurity, Fe^{++} inhibited, rather than activated, its action. Activation of papain by ferricyanide has been attributed to the formation of Fe^{++} ions.[156] After comparing the activating action of the various compounds on the basis of their iron contents, hemoglobin was found to be more effective than other inorganic iron salts.[157]

Mercaptide forming compounds were first used by Bersin. He found[59] that papain in the disulfide form was activated by p-aminophenylarsenoxide, while papain in the active thiol form was inhibited by the same substance and especially by some of its derivatives. Reduced glutathione provided protection of the enzyme and activated it even further. This has been related to the resistance of the host during arsenic therapy. Asphenamine was also found to activate papain, depending upon the nature of the substrate.[158] Other organic thiol compounds, dye stuffs, sodium thiosulphate, and various chemicals have also been studied in regard to their effects on papain.[53, 159–164]

The contradictory reports concerning the effect of various blood serums on the activity of papain have been reviewed by Walton.[165] This author found that papain, in contrast to trypsin, became slightly more active in the presence of normal human or dog serum, as determined by a photographic film method. On the other hand, it was found that the milk-clotting action of papain was prevented by the serum globulin of blood serum.[166] The liquefying action of papain on gelatin was also found to be inhibited by normal horse serum.[167, 168]

6. *Effect of Storage.* It is the general impression that papain loses its activity on storage, especially when it is in solution. Commercial preparations of papain have been reported to lose their activity in several months[7] or about one year.[169] It was recorded that papain preparations kept in sealed glass tubes for seven years had lost all proteolytic activity.[170] Fresh latex of papaya was found to split both gelatin and peptone. On keeping, the gelatin-splitting activity increased, while that towards peptone diminished.[171] Examination of various preparations of papain stored for various periods of time[172] revealed that the first change in a papain preparation with age was inactivation, accompanied by loss of the sulfhydryl substance that reacts with nitroprusside. This was followed by gradual irreversible loss of proteolytic power. It was also reported[173] that samples of papain of various grades of purity lost, on the average, about 30 per cent of their activity in 3 to 6 months, when kept at 25°C. The cruder samples of papain retained their activity better than the purer samples. On the other hand, it has been observed that a purified papain preparation retained constant activity for several months.[58] In the study of the preparation of crystalline papain,[31] it was pointed out that the enzyme was found to be relatively stable towards oxygen after several crystallizations under special conditions. Its original instability was considered to be due to an impurity. Papain crystals (Ball's) that had been preserved for years were found to have retained only one per cent of their activity,[95] however. The method of

preparing the crude papain is very important in relation to its stability on storage. As pointed out before in this paper, when sodium chloride was added to the fresh latex followed by partial removal of water in vacuum, the resulting paste was quite stable. This preparation has been kept in sealed metal tubes and in lacquered tins for 50 days at room temperature without appreciable alteration and less than 10 per cent loss in 90 days.[14]

Special studies on the stability of papain[14] confirmed that much of the activity of the papaya latex was lost during drying and on subsequent standing. Several other important points were also discussed by these authors. When papain (the moist latex paste preparation) was diluted with distilled water, there was rapid destruction of the enzyme. This destruction of the enzyme was lessened by preliminary boiling of the water or when NaCN solution was used for the dilution. Crystalline papain was much more stable, even when diluted with ordinary distilled water (not air-free). The instability of the papain preparation, especially on dilution, was found to be due to the presence of a thermostable factor which is destructive to the enzyme activity through an oxidative process.

Solutions of papain at 1:1000 concentration in one per cent sodium citrate kept under toluene at 40°C. were found to lose 50 per cent of their activity in 40 hours, as determined by the photographic film method.[165] A trypsin solution of the same strength under the same conditions showed a rapid deterioration in activity in a few hours.

VI. *Specificity and Kinetics of the Proteolytic Systems in Papain*

The evolution of the ideas regarding the specificity and the consequent classification of the proteolytic enzymes is well reflected in the studies of the proteolytic actions of papain. In the very early studies by Vines,[72] it was claimed that two enzymes, "peptase" (peptonizing protease) and "ereptase" (peptolyzing protease), could be isolated from papain by fractionation with sodium chloride. Although his finding of the acceleration of the action of papain by HCN was confirmed by later workers,[5, 6, 49, 111, 112, 125] the separation of the two enzyme components has not succeeded. Willstätter and his group[49, 112, 125] made the proposal that papain is a homogeneous enzyme because, in repeating Vines's method of fractionation of papain, the relative activity toward fibrin and peptone remained the same in all fractions. Similar results were obtained when adsorption experiments were performed. It was suggested that the activating effect of HCN is due to a "kinase" effect which extends the range of specificity of papain so that it will be able to digest both proteins and peptones. This is in agreement with a previous paper[6] and supported by a later one.[174] Both of these considered that the activation of papain by HCN is mainly due to the undissociated molecules.

The application of simple synthetic substrates in studies of the specificity of proteolytic enzymes has resulted in great progress toward understanding the action of papain. By using such substrates, it was found that the commercial preparations of papain did not attack dipeptides.[49, 175, 176] This was confirmed by Bergmann and his coworkers and additional information was obtained.[177, 178] They found that papain (crude preparation) contained

no aminopeptidase, carboxypeptidase, or dipeptidase. Any substrate with more than two peptide linkages would be split by papain, except when an adjacent amino group was present which inhibited its action. Specificity in regard to spatial configuration was also exhibited by papain. The d-form of the peptide was hardly split by papain, while the l-form was rapidly hydrolyzed. There was also indication that the side chains of the amino acids had a decided influence on the rate of the splitting.

All these activities towards the simple substrates were attributed to the action of the polypeptidase present in papain.[63] This polypeptidase fraction was distinguished from the proteinase fraction, although both are present in papain, by their different reactions towards the oxidative effect of iodine, hydrogen peroxide, and phenylhydrazine.[63, 130] The polypeptidase was irreversibly inactivated by oxidation and inhibited by phenylhydrazine. In a subsequent study,[64] in addition to confirming these findings, it was found that both of the enzyme components in papain digested gelatin. According to the specificity studies, it was first proposed that proteolytic enzymes should be classified as exopeptidase and endopeptidase. Papain contained no exopeptidase, which includes aminopeptidase, carboxypeptidase, and dipeptidase. Both the polypeptidase and proteinase were endopeptidases. They were called papain peptidase I and papain peptidase II, respectively, in conformity with this classification. The inhibition of papain peptidase I by phenylhydrazine was reversed on addition of benzaldehyde. This gives further support to the suggestion[63] that there is an essential aldehyde group in this enzyme in addition to the general conception of the thiol structure as discussed in the early part of this review. The antipodal specificity of the papain peptidase I was again elucidated in another study,[179] which also indicated that the site of action of this enzyme is directed by an immediately adjacent acylamino group. In later publications,[81, 180] the names papain I and papain II were used in place of the respective peptidases. It was found that in the presence of phenylhydrazine, which inhibits papain I, the tyrosine obtained when cattle fibrin was subjected to its action was only one-third of the total amount obtainable by the combined action of papain I and II.[81] This gives further evidence that the action of "proteinase" is not limited to high molecular weight substrates. A theory was made that papain I and papain II are inactive when associated with each other.[64] Dissociation is caused by the presence of various activators, and results in activation of these partial enzymes. This theory was supported by further experiments,[180] which are summarized in TABLE 1.

Papain I and papain II form a proteolytically inactive holopapain which dissociates into its active components under the influence of various activators and inhibitors, as well as substrates. Although this hypothesis of the two-enzyme system in papain has been given up, because these phenomena of activation and inactivation, as discussed above, were observed only when unpurified papain preparations and certain substrates were used,[131] the complexity of the proteolytic activity of papain is nevertheless well illustrated by this series of intensive studies. While the question of whether one homogeneous enzyme or two separate enzymes exist in papain remains unsettled, however, the fundamental fact that there are distinct

differences between the actions of "proteinase" and "peptonase" in papain has been observed by all these authors,[65, 66, 171, 181] as well as others.

Later studies found that simple substrates containing one peptide linkage per molecule, such as benzoyl-l-arginineamide (BAA) or benzoyl-l-lysine-amide, (BLA) were also split by papain. The kinetics of the action of papain (cysteine-activated) on these substrates were of the first order reaction.[137, 182] This is in agreement with some earlier reports on the action of papain on gelatin[111] and casein.[119] The specificity of papain and several other cathepsins, as determined by the proteolytic coefficient C_{BAA}/C_{BLA}, was found to be the same as that of trypsin and, consequently, they were all named trypsinases.[136, 137] In a recent study,[183] Ågren claimed to have demonstrated the presence of both endopeptidase and exopeptidase activities in several papain preparations. The exopeptidase was shown to be an aminopolypeptidase having a broad substrate specificity and a temperature optimum different from that of the endopeptidase component.

Table 1

Substances added	*Phenylhydrazine*	*Phenylhydrazine-HCN*	*Phenylhydrazine-Cysteine*
Effect on papain	Papain I inactive Papain II active	Papain I inactive Papain II active	Papain I active Papain II active

Purified papain trypsinase has been very carefully studied in regard to the mechanism of its activation.[132, 134, 136, 137] These investigators believe that papain trypsinase is active only in the presence of a coenzyme, the activator, with which it forms a specific dissociable compound. This coenzyme theory, as a matter of fact, has been proposed by Mendel and Blood[6] and strongly supported by Willstätter and Grassman,[49, 112, 175] but it was obscured by the oxidation-reduction theory. This latter theory is still held with strong evidence by some workers.[138] Another unusual one is the surface-action theory.[141] All these theories have been reviewed in a previous section of this communication.

A more recent study on purified papain and five other purified papainases[161] revealed that sodium thiosulfate activated the gelatin-splitting, peptone-splitting, and milk-clotting action of all these enzymes and that approximately equimolar amounts of thiosulfate and ficin or papain reacted to form an activated ferment. When fresh ficus latex was used, however, thiosulfate inhibited the gelatin-splitting and milk-clotting actions, while it stimulated its peptone-splitting activity. Because of these findings, and because thiosulfate does not reduce S—S bonds at a significant rate, this author believes that his results could be best explained by Bergmann's coenzyme theory. In this author's opinion, because different substrates were used by the previous workers (Bergmann and his coworkers used simple dipeptides,[134, 136] Winnick, Cone, and Greenberg used casein,[133, 138] and Scott and Sandrom used gelatin[141]), the different results cannot be compared. It was suggested that the discrepancy in the results of the different authors may be due to the fact that papain is a mixture of enzymes or

that it possesses various active centers which are activated in a different way.

In most of the above studies of the activity of papain as a proteolytic enzyme, the most carefully purified papain preparations were employed. The preparation must be dialyzed to remove the natural activators, which may lead to erroneous interpretation of the action of the supposedly purified enzyme.[131] The natural activator of papain was first named as phytokinase, a thermolabile substance destroyed by boiling.[176] A similar substance, zookinase, which activates both cathepsin and papain, was also found in animal organs[184, 185] These activators were shown to be reduced glutathione.[186] This was also supported by later workers,[43] but its presence in the pulp of the fruit as previously claimed[176] was denied.[67] Other workers have also recognized the presence of a natural activator in papain, but its identity with glutathione was doubted.[42, 44, 45] It was believed that papaya latex contained a thermolabile system regulating the concentration of SH compounds, which are the natural activators of papain.[187] According to a recent report,[134] a potential activator of papain was isolated by dialysis from vacuum-dried papaya latex. Its activity was retained after removal of the H_2S, but not in the case of HCN. Regarding its nature, it was apparently not glutathione, but a disulfide compound.

It was claimed that a natural inhibitor exists in papain.[41] The activating effect of Fe^{++} on papain was attributed to the removal of this inhibitor substance, since an inhibitory rather than an activating effect was observed when purified papain was used. It was also demonstrated[14] that papain preparations contained a thermoresistant destructive factor which destroyed enzyme activity, apparently through an oxidative process. The relative stability of crystalline papain has been explained on such a basis.

In contrast with a previous report[70] that digestive products of gelatin by papain gradually inactivate the enzyme by combining with its aldehyde groups, it was recently reported[188] that papain, inactivated by oxidizing agents, digested meat and became progressively more active as the proteolysis proceeded. Papain that clotted milk or digested hemoglobin very slowly, unless activated by cyanide, could digest meat rapidly without prior activation. It was believed that the natural activator was not necessarily involved, for the activation of crystalline chymopapain also took place rapidly after the addition of meat digested by pepsin.

VII. *Other Studies on the Action of Papain*

1. *Synthetic Activity.* Since the first report of enzymatic synthesis of protein material in 1886 was confirmed and the name "plastein" was given to the synthetic product,[139] numerous efforts have been made along this line. Concerning papain, Voeglein's reports of successful synthesis by this enzyme were reputable.[190–192] It was considered that, in a system consisting of papain, blood fibrin, and SH glutathione, the synthesis is favored by oxygenation in the region of neutrality when there are high initial concentration of thiol compounds and sufficient concentration of protein split products. This was supported by the report that resynthesis was favored in a system of gelatin and papain by raising the oxidation-reduction potential

through the addition of hydrogen peroxide or other suitable oxidants.[164, 193] Against this view was the finding that hydrogen peroxide inhibited the plastein formation from peptone by papain.[194] In addition, other workers were not able to reproduce Voeglein's results,[192, 195] although details of his technique[196] were carefully followed.[197] Plastein was also obtained from pepsin or papain digests of insulin[198] or egg albumin[199–201] by the action of papain. It is believed that the synthesis depends upon the concentration of the split products.

Great contributions have been made by Bergmann and his coworkers on the specificity of synthetic action of papain on simple synthetic substrates.[137, 202–205] It was found that synthesis took place when the insoluble synthetic products were removed continuously by crystallization. Activators, either the natural activator, or cysteine, glutathione, or HCN, had to be present. The optimal conditions of pH, concentrations, temperature, and activation which permitted synthesis were identical with those which are usually employed in proteolytic experiments with papain. Antipodal specificity was also manifested in the synthetic action of papain[202, 204, 205] and this has been employed to prepare d-glutamic acid from dl-glutamic acid by enzymatic resolution.[205] The important reactions, "peptide transmutation" and "coupled reaction," were also reported in these studies.[137, 203, 206]

2. *Coagulation of Blood.* Papain was first found to coagulate blood or plasma in 1937.[207] It was believed that papain, differing from trypsin, acted on fibrinogen directly. This finding has been confirmed by many other workers.[208–210] Another interesting finding deserving further study was that papain (commercial) contained a phytothrombin, which was distinct from the proteolytic fraction.[211] It was possible to destroy this thrombin by warming the solution to 60 or 70° C. for 10 to 20 minutes without causing complete destruction of the proteolytic activity toward gelatin. On the contrary, when a papain preparation was heated in the dry condition, the proteolytic activity was completely destroyed, while the thrombin was practically unaffected.

3. *Action on Other Enzymes and Materials of Biological Origin.* Papain was found to destroy urease, crude or crystalline,[212–214] and also antiurease.[215] Washed precipitates of urease-antiurease were also rapidly dissolved by papain, but only amorphous urease of lower activity was recovered from the digests.[215] Dehydrogenases from rat's liver and kidney were tested against commercial papain and trypsin, and the results showed slow destruction of the dehydrogenases at different rates.[216] When papain and pepsin were mixed in a solution, it is stated that they digested each other, the destructive agent being that enzyme which happened to be most active at the pH of the solution.[8, p. 147] Papain was found to increase the diastatic power of malt extract,[217] barley, wheat, and rye[218] and it was believed that the amylase is combined with proteins and thus liberated by proteinases.[218]

That insulin is protein in nature is in accord with the report that proteolytic enzymes, papain, trypsin, and pepsin completely inactivated insulin preparations.[219] When insulin was incubated with activated papain or pancreatin for periods too short to destroy all of its blood-sugar lowering

activity, and the digest filtered through a special filter not permeable to insulin, the filtrate was found to have no blood-sugar lowering effect.[220] This throws doubt on the existence of a fraction of the insulin molecule, which is capable of lowering blood sugar by itself.

Papain digests of a dysentery strain, Komagome B III (Ichikawa), and typhoid bacilli ("Gyo"-strain) were reported excellent antigens.[221] Papain was also found to destroy the activity of fixed virus.[222] This effect was not believed to be due to the proteolytic activity, since heat-inactivated enzymes still possessed the destructive action. Potato virus "X" was inactivated by HCN-papain at pH 4 and by trypsin and pepsin at the pHs for their optimal proteolytic action.[223] Literature was reviewed regarding the action of various proteolytic enzymes on virus preparations.[224] These authors used highly purified or crystalline enzymes and found that the elementary bodies of vaccinia were susceptible to the action of cyanide-papain, giving measurable release of amino nitrogen in two hours' incubation. The infectivity of the virus was lost in three hours and this was accompanied by changes of the morphology of the elementary bodies. There was no appreciable effect on the virus by the action of crystalline trypsin, chymotrypsin, carboxypeptidase, ficin, and cathepsin. Crystalline pepsin at pH 2 also digested the virus.

The actions of pepsin, trypsin, and papain on the "H" and "O" typhoid agglutinins were studied and the results showed that only the activated papain destroyed both kinds of the agglutinins.[225] Half and quarter molecules of beef serum pseudoglubulin and horse diphtheria antitoxin were obtained as a result of digestion by crystalline papain.[96] The quarter molecules in the digest of human γ-globulin antibodies by the action of papain or bromelin were found still to possess some antibody activity.[226] Commercial papain, activated by cysteine, was reported to reactivate rapidly the relatively pure coliphage-antibody, botulinal toxin (type A)-antitoxin, and pneumococcus-antibody.[227] Although very slow, pepsin was found to have a similar action, while trypsin and chymotrypsin have no effect at all. In a controlled study, it was reported that pneumococcus "Quellung" can be reversed by activated papain.[228]

In accordance with the early practice of using papain for the treatment of intestinal worms, it has been shown[5, 229] that papain dissolves ascaris and taenias in several hours. It was also stated that papain digested *in vitro Trichuris sp.* and *Anklyostoma duodenale*.[230] It was claimed, however, that pressed juice of *Ascaris sp.* inhibits the action of papain, trypsin, and pepsin.[231] This subject was later carefully reinvestigated, and it was found that the pressed juice of *Ascaris* (from pigs) was strongly antipeptic and antitryptic, but showed no inhibitory effect on papain.[6] A similar conclusion was drawn when a concentrated preparation of inhibitor from *Ascaris lumbricoides* var. *suis* was studied recently.[232] In contradiction to these reports is the claim that aqueous extracts of fresh and dried *Diphyllobothrium latum* and *Taenia saginata* and fresh *Ascaris lumbricoides* do not inhibit trypsin, pepsin, and papain.[233] Other workers demonstrated, however, that both commercial and crystalline papain digest live *Ascaris lumbricoides* worms *in vitro*, the crystalline enzyme being 14 times as active

as the commercial preparation in this regard.[234] On the other hand, it was reported that live tadpoles or *Arbacia sp.* eggs are not digested by papain or ficin any more than by trypsin.[235]

4. *Non-Specific Actions.* Many of the observations claimed in the reports reviewed here cannot be accounted for by the known enzymatic actions of papain. It was found[236, 237] that papain, besides its digesting and coagulating components, contained a liquefying component which was destroyed by heating to 90–95° C. After heating to this temperature, the filtrate caused an abundant precipitate when added to egg white or blood serum. This precipitating power was lost, however, when unheated papain was added to the heated substance. Papain also precipitated the mucin from Cydonia seeds.[167] Other enzymes, pepsin, rennin, trypsin, and certain other plant proteases, did not have this action and also papain did not precipitate "slimes" of some other origin. The precipitate was a stable combination, which was believed to be a result of the neutralization of the negative charge of the mucin by the positive charge of papain. Similar accounts were given when papain was mixed with the active agent of Rous chicken sarcoma[238] or influenza virus.[239] In the former case, the papain-agent complex formed was found at least as active as the original filtrate. In the latter case, however, the virus-papain complex was insoluble in water and was completely inactive. In a concentrated salt solution, the complex was dissolved, whereby the biological and antigenic activities of the virus were completely restored. The papain remained inactive unless treated with cysteine.

The heat destruction of Taka diastase was found[240] to be largely prevented by the addition of papain and, to a lesser extent by pepsin, peptone, and protein digests. Proteases previously boiled for 20 minutes were as effective as the unheated proteases. Destruction of vitamin C by boiling was also found to be inhibited by aqueous extract of papain (either fresh or previously boiled), cysteine, cystine, and some aqueous extracts of vegetables.[241]

Some interesting observations were reported by Velluz.[242] Two lethal doses of tetanus toxin or five lethal doses of ricin were inactivated instantly by 1 mg. of purified papain activated by H_2S at pH 6, but not by trypsin and pepsin. That this action was probably not proteolytic in nature was borne out by his subsequent experiments, in which he found[243] that a stable compound like strychnine formed a nontoxic substance with the purified papain. Ten mg. strychnine sulfate (4 lethal doses for guinea pig) were inactivated by 12.5 mg. of purified papain activated by H_2S, but not by 100 mg. of cysteine. Papain not activated by H_2S was less effective.

Papain was studied[244] with regard to its effect on emetics given to pigeons by various routes. It was found that papain either had no effect or, in several cases, seemed to hasten the emesis. It was stated in the same report that papain (1–2 per cent solution) seemed to strengthen the contractions of both auricle and ventricle of the isolated frog heart by lengthening the refractory period. No experimental details were given in this report, however.

5. *Pharmacological and Immunological Studies.* When a 20 per cent solution of papain was applied directly to the mesentery or injected into

the dorsal lymph sac of summer frogs, or winter frogs kept in warm places so as to bring up their body temperatures to 85° F., there was a definite inflammation of the mesentery.[245] Winter frogs gave no such response without preliminary warming. There was also a difference in the reaction of the blood of the summer and winter frogs, the former being positively chemotactic, the latter negatively chemotactic. In the same report, it was recorded that, in mice, as in summer frogs, intraperitoneal injections of papain resulted in peritoneal irritation associated with some cellular exudates.

Subcutaneous injection of sterile aqueous solution of papain into the guinea pig, rabbit, or horse produced local reactions characterized by inflammatory edema, followed frequently by a scar. Death of the animal could be caused by an injection of sufficient dose.[167] In an early report,[246] it was recorded that rabbits and dogs died five minutes after a subcutaneous injection of 0.05 to 0.1 gm. (1–1.5 grains) of papayotin.

Intraperitoneal injection of papain, 0.05 to 0.06 gm., was found fatal to a mouse of medium size, with resulting hemorrhagic peritonitis, acute splenic tumor, and congestion of kidneys, while the lethal dose by subcutaneous injections was not found to be consistent.[98]

Kubota reported[100] that there was no toxic action from injections of 1 ml. of 1 per cent papain (a refined preparation made in Japan with the trade name of Koktol) in saline intravenously and 2 ml. intraperitoneally into a mouse of 15 gm. weight, and 40 ml. intravenously and 50 ml. intraperitoneally into a rabbit of 2 kg. Injection of 5 ml. of "concentrated" solution into the abdomen of a rabbit killed the animal in 2 to 6 hours. Such rabbits showed congestion and swelling of the peritoneum and intestine, with several hemorrhagic spots.

Several other observations of the results of intraperitoneal injection of papain into animals were also made in connection with the experiments on the prevention of peritoneal adhesions. Studies made in rabbits indicated[247] that intraperitoneal injection of papain in concentrations of 1–400,000 to 1–1,000 produced no irritation. The source of the papain and the amount used were not mentioned, however. Intraperitoneal injections of "sterilized papain" of different concentrations into dogs, 1 liter per 10 kg. of body weight, gave the following results, as reported by Walton:[165, 248] papain at a concentration of 1–15,000 in saline caused some peritoneal irritation and occasional bleeding in small amounts. When a concentration of 1–3,000 was used, there was marked irritation of the peritoneal surfaces with some hemorrhage. Minimal fatal concentration was placed at 1–2,200. The hemorrhage never exceeded 14 cc. and was not considered the cause of the death. Sixteen dogs were used and the injection was given during temporary ether anesthesia in most cases. Only a few dogs were maintained under anesthesia by sodium amytal.

Injections of papain into unanesthetized dogs were made during an immunological study with papain.[259] Each of the two dogs received 20 intravenous and 4 intraperitoneal injections in the course of 97 days. Each injection consisted of 1 ml. of sterile papain, equivalent to 40 mg. of the dry powder. The intravenous injections were borne without apparent

immediate effect. In both dogs, however, the fourth injection caused a violent reaction which was anaphylactic in nature, excepting its unusually brief incubation time. Later injections, even three times the dose, gave no further reactions of this sort. The dog showed considerable discomfort for a few minutes following each intraperitoneal injection. During the course of these injections, the animals lost weight and developed hemorrhagic skin manifestations, but maintained a voracious appetite. On discontinuation of the injections, they returned to normal.

It is probable that certain of these reactions to intravenous or intraperitoneal injections of papain are manifestations of the release of histamine. Rocha e Silva[123] reported that histamine was liberated *in vitro* from a rabbit's red blood cells by the action of crude or purified papain. Synthetic compounds of histamine with amino acids were also split by papain.[250] No experiment has been reported, however, to demonstrate the liberation of histamine *in vivo* by papain.

Regarding the effect of feeding papain, it is interesting to refer to Griffith Hughes's book, "The Natural History of Barbados,"[1] in which he says: "...If hogs are for any considerable time fed with [the unripe papaya], especially raw, it is said that it will wear off all the mucous slimy matter, which covers the inside of the guts, and would in time, if not prevented by a change of food, entirely lacerate them." It was reported[246] in 1886, however, that the mucous membrane was so little affected that dogs, cats, and rabbits, which took from 2 to 5 grams of papayotin (a papain preparation by alcohol precipitation) showed no symptoms or any changes in their digestive mucous membranes. Since it was generally believed that papaya latex would have the effect of deranging the stomach when given internally, Modder did several experiments with lower animals in 1888.[251] He concluded that it did not affect the stomach in any way. Recently, Tashiro and Schmidt reported[252] the results of daily feeding of large amounts of active papain (a commercial preparation containing about $\frac{1}{4}$ of the solid of papaya juice) to various animals. The total amounts of active papain fed in two weeks on the basis of 75 kg. body weight of animals were: 134.5 kg. for mice, 4.5 kg. for rats, 604.8 gm. for guinea pigs, and 551.6 gm. for dogs. It was found that there was no harmful effect as judged by their appetite, growth, general behavior, and by the complete absence of abnormality, not only along the entire alimentary tracts, but also in various visceral and other vital organs of the fed animals. These authors also found papain harmful when applied to the skin of rabbits and confirmed the harmful effect by intraperitoneal injection in sufficient quantities.

There are only a few accounts concerning the reactions in human beings following the administration of papain. It is stated[253] that griping occasionally developed as a result of taking fresh papaya latex for the treatment of intestinal worms. Peckolt also stated[4] that intestinal inflammation may follow a relatively large dose of the fresh latex. Digestion of the gastrointestinal mucous membrane[254] or intestinal perforation[237] was feared if it were taken internally. In spite of the fairly extensive clinical use of papain, however, no experience of these complications has been reported by others.

It was claimed that, in the dog, papain had an action similar to a concentrated solution of magnesium sulfate with regard to the inhibitory effect on the tone of the distal portion of the entire biliary tract *in situ*, as well as on the isolated organs.[255]

Ågren pointed out[183] that the aminopolypeptidase from papain and that from the pyloric mucosa had similar ranges of substrate specificity. In conformity with his previous theory that the aminopolypeptidase of the hog's pyloric and duodenal mucosa may be identical with Castle's intrinsic factor, he suggested that the Castle's intrinsic factor may exist outside the body. In support of this theory, this author cited the report[256] that whole liver preparations predigested by papain gave a satisfactory response in reticulocyte counts in patients suffering from pernicious anemia, followed by a progressive amelioration of the clinical and hematological conditions with the daily administration of the preparation in an amount derived from about 47 gm. of raw liver, which is much less than the usual minimal quantity, 250 gm., for a satisfactory response in pernicious anemia. Certainly, further work is needed to elucidate this theory.

Regarding the question of whether a specific antipapain can be obtained by repeated injections into animals, there is much dispute in the literature. It was reported[257] that the toxic reaction of the guinea pig towards subcutaneous injection of papain was not ameliorated by repeated intraperitoneal inoculation of papain and that the serum obtained from such animals or from normal guinea pigs did not modify the digestion of milk by papain. Animals immunized against pancreatin did not have any protection against papain injections. Another report also indicated failure to obtain antiserum against papain.[258] In a later study,[249] sterile papain preparations were given in two dogs, 20 intravenous injections and 4 intraperitoneal injections in a course of 97 days. No inhibitory effect of the serum was observed against the proteolytic action of papain. On the other hand, definite inhibition of the proteolytic action of papain was shown by the sera from the immunized goat[246] and rabbit.[259] Recent work by Ramon[167] demonstrated that papain was changed into nontoxic "anapapain" after incubation at 40°C. with formaldehyde for one month in a solution of boric acid or phosphate, as shown by the absence of the usual reactions on subcutaneous injection into rabbits and horses. By repeated subcutaneous injections into these animals, specific "antipapain" sera were obtained, which possessed flocculating power with papain parallel to their antigelatinolytic action. Like the antidiphtheric serum, the proteolytic activity of papain could be titrated by the flocculation reaction.

The antigenicity of papain was also shown by anaphylactic reactions in sensitized guinea pigs.[259, 260] That human beings can also become allergic to papain preparations is borne out by reports of clinical cases of allergy to this substance. These cases have been reviewed by Osgood,[261] and TABLE 2 gives a summary of these reports.

In TABLE 2 are included all of the clinical reports available in the literature, but it is stated that allergy to papain is not uncommon.[263–265] It is thus seen that papain preparations are endowed with active antigenic

properties. Although sensitization is apparently due to inhalation of the dry powder in most of these cases, sensitization by ingestion or any other route may also occur.

TABLE 2

Patient's occupation	*Antigen*	*Symptoms and diagnosis*	*References*
Druggist	Papain	Vasomotor rhinitis. Reproduced by experimental ingestion or subcutaneous injection of papain. Positive scratch test.	262
(?)	Papain, pepsin, & pancreatin	Positive skin reaction even at extremely high dilution.	263
Druggist	Papain, pepsin, & pancreatin	Vasomotor rhinitis and asthma. Worse on dispensing these digestive enzymes. Skin test highly positive. Swelling of lips on tasting a solution of papain. Improvement following treatment by desensitization.	264
Druggist	Papain	Severe asthma on exposure to papain. Unconscious on two occasions as a result of acute attack.	264
Druggist	Papain	Urticaria, angioneurotic edema of face, and dyspnea after ingestion of papain.	264
(?)		Cramps and diarrhea immediately after taking a dose of Essence of Caroid. Repetition of the dose refused. Coincidence not ruled out.	264
Druggist	Papain, pepsin, & pancreatin	Vasomotor rhinitis when working with these digestive enzymes. Skin test ++ to papain. No symptoms following ingestion of large amount of papain.	265
Housewife	Papain	Caroid powder was used for treatment of sloughed wound of her leg following ligation of varicose veins. Coryza and asthmatic attacks developed when her husband began to use Caroid tooth powder about two years later. Severe skin reaction to Caroid. Definite passive transfer on a nonallergic individual. Negative precipitin test. No further attacks after removal of the Caroid tooth powder.	261

VIII. *Assay of Papain*

Various methods have been used to measure the activity of papain. The difficulties of accepting a single method as satisfactory for the assay are clearly shown by the diversity of opinion in the literature concerning the

optimal pH and temperature, the wide range of specificity regarding the nature of substrate, and the various effects of impurities and different activators. As a matter of fact, various assay methods for papain have been developed in close association with the expansion of the knowledge of papain. Most authors have chosen a method of their own, however, and therefore their results, at times, are seemingly not comparable. Not only because of the active scientific research in this field, but also because of the increasing use of papain for various purposes, a demand exists for an accurate method for measuring the activity of this enzyme.[11, 15, 110, 266, 267] Although rapid progress in the understanding of papain has led to the establishment of many valuable assay methods, an official method of standardizing papain preparations proposed for different uses has met great difficulties from a scientific point of view.[268]

Methods for the assay of papain activity have been numerous and the principles of the quantitative measurement are different in many ways. No details of the procedures will be described in the present review and only the key references will be given.

Digestion of ground raw meat has been used in some earlier attempts to ascertain the papain activity of preparations from different parts of the plant by comparing the amount of undigested meat residue after six hours' incubation at 52° in 0.3 per cent HCL.[12] The use of such an acid medium for the digestion has been greatly criticized.[83, 266] Some difference in opinion has been expressed in using raw meat or cooked meat as the substrate. One report stated that raw meat was better digested by papain,[269] while recent workers found no difference between the digestibility of cooked and uncooked meat.[88, 270] Owing to the impossibility of obtaining meat at different times that approaches a uniform digestibility, there was a wide variation in the percentage of digestion.[271] As an alternative method, this author obtained satisfactory results by using dry steak powder as substrate instead of the raw meat.[271] In the 8th edition of National Formulary,[272] the meat powder method has been adopted, using a digestion period of two hours instead of six hours. The result should always be compared with the results obtained using the reference papain, which is obtainable from the Chairman of the Committee on National Formulary. In connection with the use of meat as the substrate, it is interesting to note that no activator is recommended for the assay[269, 273] and that it has been shown that there is no need of such an activator to be added to the digestion mixture under these conditions.[188]

Egg albumin was used as substrate for the assay of papain, by measuring either the volume[273] or the weight[110] of the undigested residue. The digestion time of fibrin by papain was also employed.[270] Fibrin is considered to give low results, however, as compared with other substrates, probably because it adsorbs the enzyme.[120]

Casein has been used for the assay of papain in a number of ways. The extent of digestion of casein was ascertained either by polariscopic examination of the filtrate,[267] alcoholic titration,[120] formol titration,[27, 31] or by determining the first disappearance of the turbidity on addition of acetic

acid to a series of test tubes containing definite amounts of casein and varied amounts of the enzyme.[274] In the first official method for the assay of papain, casein was also used.[275] The use of sodium bicarbonate in the assay process, however, brought the reaction of the digestive mixture to pH 9.2, which is considered unfavorable for papain digestion.[173]

Gelatinolytic action of papain has been noticed in the very early studies. Determination of papain activity by formol titration of the digestion products of gelatin is commonly used.[173, 270] The effect of papain on the phenomenon of mutarotation[276] or on the viscosity[277] of the gelatin solution has also been used with satisfactory results. Determination of the minimal amount of papain needed to cause effective gelatinolysis is used in another laboratory.[167] The use of gelatin as substrate for papain should be extremely cautious, however, since impurities, such as certain heavy metals often contained in gelatin, have great inhibitory effect on papain activity.[120, 152, 153] A photographic film method has been claimed to be very sensitiev in assaying papain solutions of high dilutions.[270] The principle of this method is based upon the photometric reading of a sensitized photographic film after the digestive action by papain on the gelatin emulsion.

Condensed skimmed milk was also used as substrate for assay of papain by determination of the undigested protein.[123] Although many studies have been made in regard to the milk-clotting action of papain, a good method for the assay of papain by the utilization of this property of the enzyme was developed only recently.[27] Dried whole milk is used to prepare the substrate, and clotting time is measured to ascertain the strength of the enzyme. The enzyme should be fully activated with hydrogen sulfide or other suitable activator and a short clotting time (1–5 minutes) must be used to avoid oxidation. Commercial papain was used and its milk-clotting activity was parallel to its activity against casein. This conclusion was confirmed with crystalline papain when parallel milk-clotting and hemoglobin-digesting experiments were made.[31] For this reason, and because of the simplicity of the procedure, this method has been used by many workers. It has been pointed out that each dried milk preparation should be standardized by a reference sample of papain, because occasional preparations of dried milk varied considerably from the average in clotting time.[14, 31]

It is reported[278] that the hemoglobin method used for assay of trypsin can also be used for measuring papain activity, provided that papain is properly activated with cyanide and there is sufficient cyanide in the hemoglobin solution. Denatured hemoglobin at pH 7.4 is digested for 5 minutes at 25°C.[278, 279] or 30°C.,[31] and the split products not precipitated by trichloroacetic acid are estimated colorimetrically with a phenol reagent. By this method, only the first stage of digestion is measured, for hemoglobin needs to be only slightly digested for the digestion products to be nonprecipitable by trichloroacetic acid. It is not known whether all samples of commercial papain give the same activity curve.[279] An important advantage of this method, however, is its reliable and reproducible substrate.

Simple synthetic compounds have been used for the study of papain activity. One of the frequently used substances is hippurylamide.[31, 124, 268] The rate of splitting of this substance by activated papain is measured by a formol titration technique.

The activity unit of papain has been defined according to the substrate employed. Thus, according to Balls and Lineweaver,[31] one milk-clotting activity unit of papain or [Pa.u.]$^{M.}$ is the amount of papain that will clot 5 cc. of standard milk preparation at 30° in one minute. For digestion of hemoglobin, the initial rate of digestion at 25°C.[278] or 30°C.[31] by one activity unit [Pa.u.]^{Hb} is such that there is produced per minute in 6 cc. of digestion mixture an amount of color-producing substance not precipitable with trichloroacetic acid that gives the same color with the phenol reagent as one milliequivalent of tyrosine. For hippurylamide, one activity unit [Pa.u.]^{HA} is defined as the quantity of enzyme which causes an increase in the titration representing splitting of hippurylamide at the initial rate of one milliequivalent per minute. The activity of papain preparations may be expressed as $[\text{Pa.u.}]_{\text{mg.P.N.}}$ or $[\text{Pa.u.}]_{\text{ml}}$, in which the subscripts refer to the number of units per mg. of protein nitrogen or number of units per ml. of enzyme solution.

TABLE 3

Year	*Quantity exported from Ceylon to U. S. A., pounds*	*Reference*
1911	550	15
1912	2,048	15
1913	13,078	15
1938	173,000	280, 281

The British Pharmaceutical Codex[275] official method for papain preparations requires that the amino acids liberated from casein by one gram of papain require not less than 20 ml. of 0.1 N sodium carbonate for neutralization. The high pH of the digestive mixture was criticized and a method using gelatin for the substrate at pH 5 was suggested as a substitute for the B. P. C. method.[173] According to the 8th edition of the National Formulary,[272] a papain preparation is required to possess a digestive activity not less than that of the N. F. Reference papain, as determined by the method described before.

IX. *Practical Uses of Papain*

Although papain was used as a medicine in early times[4] and was brought to America as a cure for dyspepsia,[10] the amount used for such purposes is very small compared with the total quantity consumed. According to the Chemical Division of the U. S. Department of Commerce, the importation of crude papain by the United States increased fourfold from the year 1932 (54,000 pounds) to 1938 (223,000 pounds).[280, 281] Ceylon is the largest exporter of papain to the United States, as shown by TABLE 3. It is finding

its greatest use in the manufacture of "food tenderizers"[22, 280] and for some other industrial purposes.

1. *Commercial Uses.* The use of papain for tenderizing meat has been practiced from time immemorial by the natives of tropical areas, either by boiling the meat with the unripe fruit of the papaya tree[1] or by wrapping the meat with its leaves.[4] This received experimental confirmation in 1878.[3] Meat rubbed with a slice of green papaya also becomes tender as a result of the action of papain.[282] It is stated[88] that in the usually recommended procedure for tenderizing meat, unground meat is painted with a solution of papain and then cooked. These authors found, however, that papain would not penetrate very far into the meat under such conditions, and the papain on the surface would be inactivated by heat before much digestion could take place. Their results indicate that 80°C. is the most favorable temperature for the rapid digestion of ground meat by papain during a 30-minute period and that ground beef is much more rapidly digested. Previous cooking did not increase the rate of digestion. Papain preparations have been sold under various trade names for tenderizing meat.[283, 284] According to one report,[285] meat tenderizers are prepared in Florida by dissolving imported papain in slightly acidified water. Two samples showed no milk-clotting activity unless first activated. Analysis of a sample of one tenderizer revealed that it contained 19 gm. of papain and 38 gm. of U.S.P. lactic acid per gallon of water.

Valuable nutrient media for many kinds of bacteria have been made from papain digests of various meat proteins.[286–289] It is claimed[290] that papain-digested meat medium meets almost all the requirements for large scale production, since it is inexpensive, simple, and quick and gives a uniform product. It is also reported[291] that peptone for bacteriological cultures can be prepared from casein by enzyme hydrolysis. Three types of casein peptone have been made by the action of three different enzyme preparations, pepsin, pancreatin, and papain. They are very simple to make in highly reproducible form, give a perfectly clear and almost colorless solution in water, and produce no precipitate when added to meat infusion.

After a simple papain digestion, almost twice the amount of oil was obtained from tuna liver by extraction and the oil contained several times the concentration of vitamins A and D_2 compared with normally extracted oil.[292]

The stability of beer is increased by papain, especially in the presence of glutathione and ascorbic acid.[293] Papain has been used in the brewing industry for many years for making "chill proof" beer, since it prevents turbidity on cooling by digesting the traces of protein dissolved in the beer.[22] It has been estimated that 80 per cent of American beer is thus treated.[22, 294]

Papain is used in the tanning industry for bating skin and hides.[294] It is also used in the textile industry to make wool and mixture fabrics of better quality.[295] The wool treated with papain is white and clean and dyes evenly to bright shades.[296]

2. *Therapeutic Uses.* Besides being used for tenderizing meat in the early days, the milky juice of the *Carica papaya*, or papain, has been used for many therapeutic purposes.

A. *Medical Uses.* According to the statements in the Pharmacopeia of India,[253] the anthelmintic properties possessed by the milky juice of the unripe fruit of the papaya tree were first shown in the 17th century by Hernandez. Its efficacy as an anthelmintic was later confirmed by others. The following mode of administration employed by LeMarchand is considered desirable. A tablespoonful of fresh papaya latex is thoroughly mixed with an equal amount of honey. Three or four tablespoonfuls of boiling water are then added gradually. When sufficiently cool, the whole is taken at a draught, followed by a dose of castor oil two hours later. A smaller dose is given to children. Its effect is said to be mainly on the round worms and very little on *Taenia.* A similar statement appears in a later book.[297] Modder has employed this treatment successfully in both ascariasis and ankylostomiasis.[251] In the Bengal Dispensatory, 1843, it is stated that no obvious effect was observed when a dose of 20 to 60 drops of the milky juice was used.[298] This was considered to be due to insufficient dosage.[253]

In 1879, Peckolt claimed extraordinary results in treating intestinal worms, especially ascarides, with his papayotin preparation.[4] Success was also reported[229] in using papain for treating patients infested with *Ascaris* and *Taenia.* Parallel *in vitro* experiments demonstrated the actual rapid digestion of these worms by the enzyme. Several other reports gave a similar conclusion.[299, 300] According to another report,[230] this kind of treatment of intestinal worms had been a common practice by the physicians of that time. Although the digestion of the live worms by papain *in vitro* has been confirmed recently, as discussed before, no other scientific accounts of the use of papain as an anthelmintic have appeared in the literature. There are several reports, however, of the successful use of a papain-like enzyme, ficin, in treating patients of ankylostomiasis[230] and trichuriasis.[301, 302]

Although papain has been consumed in this country in recent years mainly for tenderizing meat,[22, 280] it was first imported as a medicinal for the treatment of chronic dyspepsia.[10, 15] It is stated[10] that there are no dyspeptics in the tropical home, but its use along similar lines is by no means unknown: "Some of these people are great gluttons; they gorge themselves until the skin on their distended stomach is stretched to its utmost. It is certain that no human being could digest the kind of food and the enormous amounts they consume without the kindly aid of the papaw fruit to assist digestion."

It is interesting to note that in the first account, in 1874, of the digestive action of papaya latex on protein food, Roy suggested the administration of a few grains of the dried latex after meals in cases of indigestion.[2] Soon after Wittmack's report,[3] it was also speculated that it would be a valuable addition to our means of treating various kinds of dyspepsia and would be a cheap substitute for pepsin.[303] As a matter of fact, Peckolt reported his work in the same year on "papayotin," made from the fresh latex of papaya.[4] He used it many times in ailments of the stomach where pepsin was indicated with good results. Either the glycerine solution or the dried papayotin, in doses of 0.2 to 0.25 gm. at each meal-time, was employed. Bouchut used his papain preparation in all forms of dyspepsia and gave it in syrup to children suffering from chronic diarrhea, with good results.[304] Papaya

latex was also used to aid digestion.[251] Pemberton reported satisfactory response to treatment with Caroid in 50 cases of infantile diarrhea.[305]

By 1880, different pharmaceutical preparations of "papain or vegetable pepsin" had been put on the market.[306] Apparently, such preparations attracted a great deal of attention among practitioners in this country[307] and were in active demand in those years.[308] However, many preparations on the market were found to be very low[309] or even worthless in digestive power.[310, 311] Popular papain preparations were Caroid and Essence of Caroid.[312, 313] The Council on Pharmacy and Chemistry recommended in 1914[269] that these be considered no further until the manufacturer was in a position to furnish products of constant digestive power.

Papain has been in fairly constant demand.[173] Its importance has been recognized by inclusion in the British Pharmaceutical Codex,[275] although the method of assay was considered inadequate.[173]

Even a short section on papain did not appear[272] in this country until the latest edition (1948) of the National Formulary. It is stated in the Epitome of the Pharmacopeia[314] that the medicinal value of papain is questionable. In a recent report,[315] however, it is mentioned that papain is available from druggists in various forms. This author treated 17 consecutive patients, suffering from esophageal obstruction due to meat impaction, by oral ingestion of papain (15 gr. every hour) or Caroid (1 teaspoonful in $\frac{1}{4}$ glass of water, given in sips for high obstruction). Sixteen of these patients were promptly relieved of their obstruction. The other patient, unable to retain the medicine, required operative removal.

It is said that papaya latex has strong emmenagogue action when taken internally and, in large doses, may cause abortion,[297] but there has been no such report in the literature. Moreover, the latex has been used in treating patients with "splenomegaly and hepatomegaly" with very good results.[163, 316]

Use of papain by local application has been practiced since the early days. Some common diseases thus treated with favorable results were diphtheria[237, 246, 254, 297, 308, 317] and croupous angina.[237, 318] The method recommended consisted essentially of frequent application of a 5 to 10 per cent papain solution in equal parts of water and glycerine to the affected area. Various lesions, such as syphilitic ulcers of the tongue and throat,[319] yaws,[10] chronic eczema and psoriasis,[251, 320] ringworm,[251, 297] and skin spots and freckles[4, 237, 251] have also been treated by the local use of papain.

Recently, as an adjunct to hyaluronidase treatment in human infertility, papain, about 5 mg. in powder form, has been used to pack the cervical canal in patients with endocervicitis, on the basis of the fact that papain liquefies cervical mucus. Two cases thus treated resulted in pregnancy.[321]

B. *Surgical Uses.* Papain solution has been used for the removal of cerumen impacted in the ear.[322] Recently, cases of chronic purulent otitis media have been treated successfully with Caroid solution.[323, 324]

In 1888, Modder tried the local application of papaya latex in several cases of sloughing ulcers, and he noticed that it hastened the separation of the slough and brought on a healthy action in a very short time.[251] Kilmer

witnessed the striking effect that resulted from the use of a paste of papaya latex by natives as a dressing for a badly infected wound.[10] Similarly, Glasser reported 58 cases of sloughing wounds treated with active papain.[325] Several reasons for the excellent results of using this treatment in removing slough and promoting the healing of wounds were discussed. In another report, papain-cysteine-salicylate solution was used for "enzymatic debridement" in six cases of burn.[326] The eschar was easily removed and rapid epitheliation took place after cod liver oil dressing.

In 1880, Bouchut reported the digestive action of papain on the tumor growth of some patients.[327] He injected the solution into the tumor in one case of adenoma and three cases of cancer of the breast. Each time it was followed by great pain and marked fever, but the tumor was softened and converted into abscesses which healed after open drainage. The liquid aspirated from the softened cancerous tissue was analyzed and was found to contain true peptone. Similar observations were reported by Branch in 1906 in four cases of scirrhus in the cicatrix of amputated breast.[328]

A new treatment of ganglion by injecting a water suspension of Caroid powder was recently reported.[329] It was claimed to be a practical, atraumatic, and safe procedure. When the same treatment was employed in another case of ganglion, however, a tragic result, with severe infection and wide spread necrosis, was described.[330] This author made cultures of the Caroid he used, as well as two other samples, and found that they were all similarly heavily contaminated with *Escherichia coli*, *Bacillus subtilis*, *Micrococcus pyogenes* var. *albus* (*Staphylococcus albus*), and non-hemolytic streptococci.

As discussed in a previous section, many studies have been made on the effect of papain solutions injected into the peritoneal cavity. This was soon applied to the problem of prevention of post-operative peritoneal adhesions, especially in individuals with "adhesion diathesis."[331] Extensive work has been performed, but the results are controversial. In studies along this line, one of the important requirements is sterilization of papain for injection. The best method worked out was to filter a glycerine solution of papain through a Berkfeld filter and then to precipitate it with alcohol. The sterile dry powder was then sealed in ampoules.[248, 270] Through the effort of this author, much has been learned concerning the toxicity and behavior of papain in the peritoneal cavity. The findings which encouraged the later workers to try papain for the purpose of preventing intra-abdominal adhesions were essentially the slight accentuation of papain activity by the normal serum and the prolonged activity in the peritoneal cavity even at high dilutions.[165]

Experimental production of peritoneal adhesions and its prevention by papain solutions has been claimed successful by several investigators.[100, 331–334] Clinically, large series of patients have been tried[331, 334, 335] and good results were obtained. In another study at about the same time, however, it was concluded that papain was of no value in the prevention of experimental postoperative adhesions.[336] In a later clinical study of a smaller series (72 cases), divided into "papain" and "no papain" groups of nearly equal

numbers of cases,[337] the authors followed closely the technique described by Ochsner and Storck but were not convinced of any special advantage in the use of papain, however. They stated that, although the results of 88.8 per cent of the 224 cases in the Ochsner and Storck's series[335] were considered satisfactory, the evaluation of their results was difficult, since there were no statistics of a similar series of cases in which papain was not employed.

In a later experimental study with 26 rabbits,[338] Donaldson reached the conclusion that he was unable to demonstrate that papain was effective in preventing the reformation of adhesions. He affirmed that the method he used was satisfactory in producing fairly uniform results of peritoneal adhesions, while the previous workers employed methods which could hardly be quantitative. The possible divergence in technique is highly important in the interpretation of the experimental results.

Summary

Papain is the dried whole latex from the green papaya, fruit of a tropical plant, *Carica papaya*, Linn. It is a white or cream colored powder with characteristic pungent odor. Enzymatic activities of this product have been extensively studied in the last 70 years. The most noticeable activities are the proteolytic and the milk-clotting activities, which are probably manifested by the same enzyme component in papain. Lipase and lysozyme activities, moreover, have also been demonstrated in papain preparations. Amylase activity is only shown by the fresh latex.

Various methods have been used for its purification. Two highly active crystalline enzymes, crystalline papain and crystalline chymopapain, have been recently isolated from the whole latex product. They have been found to be equally potent with respect to milk-clotting activity, but the former shows twice as much proteolytic activity as the latter. The latter is present in the latex in considerably greater quality than the former, however, although their exact proportion is not known. They are both protein in nature. The molecular weight of the crystalline papain has been determined as about 27,000.

Many studies concerning the proteolytic activity of papain have been made with crude or partly purified preparations. This accounts in part for the controversies in the literature. The chemical nature of the active proteolytic group of papain has been a long debated subject. It seems generally believed that the sulfhydryl group is essential, but probably an aldehyde group is also important. Under appropriate conditions, papain digests proteins to the stage of amino acids. Whether or not the protein is denatured before being split by papain has been carefully studied, but the results are still considered inconclusive.

Papain as a proteolytic enzyme is remarkably thermostable. When in dry form it resists destruction by heat at 100°C. for three hours but its solution is inactivated by heating for 30 minutes at 82.5°C. While papain is proteolytically active even at 10°C., its optimal action is observed at 70°C. Papain is active within wide zones of pH, acid, neutral,

or alkaline. Crystalline papain is rather stable between pH 3 and pH 12, while crystalline chymopapain is stable even at pH 2. The pH optimum for the proteolytic action of papain varies according to the nature of the substrate; pH 5 for gelatin, peptone, and synthetic simple substrates, near neutrality for fibrin, casein and hemoglobin. There is an indication that the optimal pH for the initial rate of digestion of certain protein substrates is different from that for the complete digestion.

One of the notable characters of the proteolytic activities of papain is its reversible oxidative inactivation. Papain is inactivated by various oxidizing agents, even atmospheric oxygen, and reactivated by reducing agents, including hydrocyanic acid, one of the antiseptics used in the earlier studies of papain. These reversible reactions have been interpreted on the basis of the thiol nature of the enzyme. In recent studies using strict anaerobic technique and highly purified enzyme preparations, it was concluded that papain exists in two inactive forms, a- and B-papain trypsinases, which are interconvertible by processes of oxidation and reduction. However, activation of the enzyme is achieved not by such processes but by the presence of a co-enzyme, either hydrogen sulfide, hydrocyanic acid, cysteine, or reduced glutathione. The nature of the co-enzyme determines the proteolytic coefficient of the enzyme. Opinions differing from this conception have been discussed.

Inactivation of papain is effected not only by various oxidizing agents, extreme acidity and alkalinity, and very high temperature, but also by excessive exposure to ultraviolet light of certain wave length.

Regarding the effect of other substances on the proteolytic action of papain, the literature has been reviewed with emphasis on certain relatively more important chemicals and some biological materials. The effect of ascorbic acid on papain activity is still controversial. Iodoacetic acid causes irreversible inactivation of papain by destruction of its sulfhydryl group or some other essential group. Certain metal ions, such as copper, gold, silver, zinc, cadmium, and mercury, are inhibitory to the action of papain, while other metals have no effect. Such observations have led to the important theory that the action of papain activators is to remove these toxic metal ions. This theory is not supported by other workers, however. The effect of mercaptide-forming compounds on papain has been briefly discussed. Reports concerning the effect of normal blood serum on papain are still controversial.

The proteolytic activity of papain deteriorates on storage, especially when it is in solution. Its instability has been ascribed to the effect of oxidation and to the presence of impurities, which may have either an inhibitory or a destructive effect on papain. The destructive factor is thermoresistant and destroys papain in solution, apparently through an oxidative process. Natural activators, similar to reduced glutathione in chemical nature, are also present in ordinary papain preparations.

Papain contains a complicated proteolytic system. "Proteinase" and "peptonase" theories have been given up by some authorities after ex-

haustive studies. The question still remains whether papain is a mixture of enzymes, or a single enzyme, possessing various active centers which are activated in different ways.

Papain was formerly classified in the group of proteinases. It has recently been considered as an endopeptidase, according to a new classification by Bergmann. The specificity of papain and some other cathepsins was found to be the same as that of trypsin, and the name trypsinase was therefore given to this class of enzymes. A coenzyme is essential for its proteolytic activity. The rate of splitting of a peptide bond by papain is inhibited by an adjacent amino group and much influenced by the nature of the side chains of the amino acid and stereochemical configuration. The site of action of the enzyme, at least the "peptonase" fraction, is directed by an immediately adjacent acylamino group.

Papain exhibits synthetic activity under certain conditions. Blood is coagulated on addition of papain, due to its direct action upon fibrinogen. Under appropriate conditions, papain also destroys certain other enzymes, insulin, certain bacteria, and viruses, as well as antibodies. Worms like *Ascaris*, *Taenia*, hookworm, and *Trichuris* are digested by papain. In *Ascaris*, antipepsin and antitrypsin are demonstrated, but not antipapain. Some other actions of papain, which are not explained on the basis of its proteolytic activity, are also described.

Parenteral administration of papain has been found toxic to animals, but large doses by ingestion are probably not harmful. Both animals and human beings may be sensitized to papain. Several clinical cases have been recorded. A possible relationship of papain to the Castle's intrinsic factor has been discussed.

Methods of assay for papain have been discussed and the definitions of the different activity units of papain have been given.

Papain was first brought to America as a medicinal, but it is now also used for numerous commercial and industrial purposes, especially for making food tenderizers.

As a digestive enzyme, papain has been used in medicine for various conditions. It has been administered internally as an anthelmintic and for aiding digestion. Externally, it has been applied for certain ulcerative and various other skin lesions. It has been injected into certain tumor growths with good results. Intraperitoneal application of papain has been used with apparently good results for the prevention of postoperative adhesions, but the data have been considered doubtful.

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STUDIES OF METHODS FOR MEASURING THE PROTEOLYTIC ACTIVITY OF PAPAIN

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The fresh latex obtained from the green fruit of *Carica papaya* L. contains powerful proteolytic enzymes, which are used in the form of the dried whole latex, called papain. Since the utility of papain depends upon its enzyme content, various methods have been employed to measure its proteolytic activity. Unfortunately, they have not all given comparable results. Papain, along with a method for its assay, was recently admitted to the 8th edition of the National Formulary and thus became an official drug in 1946. It was soon found that the official method of assay gave results that were different from those obtained by other methods employed in these laboratories, so that either a new method of assay for the proteolytic activity of papain had to be developed or means found for reconciling the results of existing methods.

In all methods, papain is allowed to digest a protein or peptide and the extent of such digestion is then measured. The methods can be divided into three main groups, depending on the manner of measuring the amount of digestion, *i.e.*, those depending upon the measurement of undigested residues, those depending upon the measurement of liberated amino groups, and miscellaneous methods based on the measurement of such things as the optical rotation of digests, the length of time required to clot a standard milk solution, the color produced by certain reagents on filtered digests, the increase of soluble solids, *etc.*

In the first group belong such methods as the National Formulary method,[1] which is based on the volume of undigested residue left after digesting a suspension of powdered dry beef. This method is probably derived from the prior work of Graber,[2] Adams,[3] and Unwin,[4] who reported the use of both fresh and dried beef as the protein substrate. Maher and Wirth[5] proposed an improvement of the N.F. method. Rippetoe[6] and Heyl, *et al.*[7] employed heat-coagulated egg albumin, the former measuring the volume of undigested residue and the latter weighing the undigested protein after washing and drying. Pratt[8] used condensed skimmed milk as his substrate and weighed the amount of protein precipitated by $CuSO_4$ and acetic acid solutions. Siewert[9] reported a method similar to the U.S.P. method for pancreatin,[10] in which tubes containing casein solution are incubated with varying amounts of papain. Alcoholic acetic acid solution is added and those tubes developing precipitates are distinguished from those remaining clear.

The method presented in the Methods of Analysis of the A.O.A.C.[11] belongs in the second group. It employs a casein solution buffered with citrate at pH 5. The liberated amino[12] groups are titrated with 0.1 N alcoholic KOH after the addition of large volumes of alcohol to the digest. Bergmann and co-workers[13–15] made extensive use of synthetic peptides as substrates for studying the proteolytic activity of papain, the extent of

hydrolysis being followed by titration with alcoholic KOH after the addition of alcohol to the reaction mixture. The method included in the British Pharmaceutical Codex[16] uses an alkaline casein solution for the protein substrate with the liberated amino groups being determined by formol titration with 0.1 N Na_2CO_3. Formol titrations have been used by a number of investigators to determine the extent of hydrolysis of various proteins. Iyengar[17] proposed a standard method of assay based on the formol titration of gelatin digests. Scott and Sandstrom[18] and Hoover and Kokes[19] employed the Van Slyke amino-N method to follow the extent of digestion of proteins, the former using gelatin and the latter using casein as their substrates.

Greenberg and Winnick[20] developed a method based on the increase in optical rotation of casein digests. The digestion mixture is treated with trichloracetic acid and filtered. The increase in optical rotation of the filtrate is proportional to the amount of hydrolysis. Anson's hemoglobin method[21] is based on the colorimetric estimation of the split products, not precipitable by trichloracetic acid, that are formed by the proteolysis of denatured hemoglobin. Balls and Hoover[22] developed a method based on the length of time required for a quantity of papain to clot a standard milk solution. The Argentina Pharmacopoeia[23] contains a method that estimates the activity of papain by determining the amount of soluble solids contained in an aliquot of the filtrate from a hog fibrin digestion mixture. The proteolytic activity of papain has also been estimated by incubating tubes of gelatin solution containing varying amounts of papain, chilling, and then noting which tubes are gelled and which are liquid.[5]

Present Studies

Since it was felt that a method employing a complete protein would give most useful data, no work was done with methods using Bergmann's synthetic peptides.[13–15] Casein, egg albumin, and crystalline bovine serum albumin were chosen as representative whole proteins that might be suitable for use as substrates. Dried beef powder was included, since we felt that it had not been investigated sufficiently. Gelatin, although a partially hydrolyzed protein, was also included because of its ready availability and ease of handling. Although methods depending upon the comparative volumes of undigested residues are generally not trustworthy, they usually have the advantage of requiring simpler apparatus and techniques. On the other hand, methods depending upon volumetric principles to measure the extent of proteolysis are almost as simple and generally much more accurate. Both types of methods, however, were investigated in studies dealing with the effects of pH, enzyme concentration, temperature and length of time of digestion, substrate concentration, and, in one case, different lots of protein, on the proteolytic activity of the enzyme. Although papain's milk-clotting activity is considered by some as distinct and different from its proteolytic activity,[5] a modification of the Balls and Hoover method[22] was used to measure, for comparative purposes, the activity of the various samples of papain used in this study.

Methods

Beef Digestion. The N.F. method[1] was used as described, with the addition of a blank control containing dried beef powder but no papain. The difference in the volumes of residue between the blank control and the digests is a measure of the amount of digestion which is reported as ml. of beef digested.

The Maher and Wirth modification[5] was also used as described by the authors, employing 18-hour-old filtered papain solutions. Better results were obtained, however, by using freshly prepared suspensions of papain in cold, boiled water. These results are reported as ml. of beef digested.

The flocculent nature of the residue from the N.F. and the Maher and Wirth methods led to the following modification of the latter method: place 0.5 gm. of powdered dried beef into a 15 ml. graduated centrifuge tube, add 7 ml. of water, suspend the beef by stirring with a small glass rod, add 1 ml. of papain suspension representing 0.5 mg. of papain, and stir again to mix. In a similar manner, prepare a tube containing 0.5 gm. of beef powder and 8 ml. of water, but no papain, to serve as a blank control. Incubate the tubes in a water bath at 70°C. for 2 hours, remove from the bath, add water to the 15 ml. graduation, and then centrifuge at 1800 R.P.M. for 30 minutes. The difference in volume of residue between the control and the digest tubes is a measure of the amount of digestion, which is reported as ml. of beef digested.

Trichloracetic Acid Precipitation. Trichloracetic acid is extensively used as a protein precipitant. In two papain assay methods,[20, 21] it is used to remove interfering undigested protein from the hydrolysis mixture. A method based on the comparative volume of precipitate formed by treating papain digests with trichloracetic acid was investigated. The general procedure was as follows: measure 5 ml. of a 5 per cent (6 per cent in the case of casein) buffered protein solution into a 15 ml. graduated centrifuge tube, place the tube in a constant temperature water bath at 40°C., and allow the temperature of the protein solution to reach equilibrium. Add 1 ml. of papain suspension, invert to mix, and replace the tube in the bath. In a like manner, prepare a tube containing 5 ml. of the protein solution and 1 ml. of water (no papain) to serve as a control. Digest for 30 minutes, then add 5 ml. of 10 per cent trichloracetic acid solution, stir with a small glass rod to break up any curds of precipitated protein, and centrifuge at 1800 R.P.M. for 30 minutes or until the precipitate has packed to a constant volume. The difference in the volume of precipitates between tubes is a measure of the extent of digestion. The results are reported as ml. digested. Casein, egg albumin, or crystalline bovine serum albumin were used as substrates in this method.

Formol Titrations. Gelatin, casein, and egg albumin were investigated as possibly suitable substrates for formol titration of the extent of digestion. The following generalized method was used throughout: measure 20 ml. of buffered protein solution into a suitable container, add 10 ml. of papain suspension, and incubate in a water bath at the directed temperature for the required length of time. At the end of the digestion period, add 30

ml. of freshly neutralized (to phenolphthalein) formalin (38–40 per cent formaldehyde) solution and titrate with 0.1 N NaOH to a phenolphthalein end point. A blank control is also run containing 20 ml. of the protein solution and 10 ml. of water instead of the papain suspension. The difference between the volumes of 0.1 N NaOH required to neutralize the control and hydrolysis mixture is a measure of the extent of hydrolysis, reported in milliequivalents of $-NH_2$.

Milk Clotting. The method of Balls and Hoover[22] was used, except that 25 ml., instead of 10 ml., of the buffered, reconstituted milk was employed. Since the end point described by them is not sharp, the following technique, developed by N. F. Blau* in the laboratories of the American Ferment Co., Trenton, N. J., was used: measure 25 ml. of the milk solution into a 40–50 ml. test tube and place in a constant temperature water bath at 40°C. When the milk solution in the tube has reached the bath temperature, add 1 to 3 ml. of papain suspension, stopper the tube with a rubber stopper, shake, and return to the bath. Rotate the tube intermittently in a horizontal position below the surface of the water in the bath so that its walls are coated with a thin layer of milk. It will be noted that the milk readily drains from the sides of the tube whenever the rotation is stopped. Just prior to clotting, this milk layer thickens and fails to drain from the walls of the tube. The rotation of the tube should be stopped at this point and the thickened layer of milk closely observed. The actual clotting is readily noted because the continuous film of milk on the walls of the tube becomes discontinuous and pebbly almost instantaneously. In our hands, this is a much easier and more reproducible end point than that described by Balls and Hoover.[22] The length of time from the addition of the papain suspension to the clotting of the milk is recorded. The results are expressed as milk-clotting units of activity per gram (UA/gm.), a unit of activity being the amount of papain necessary to clot 25 ml. of the milk solution in 1 minute at 40°C. Units of activity per gram are, therefore, equal to $1000/(E \times t)$, where E is the weight of papain in milligrams that clots 25 ml. of the standard milk preparation in t minutes.[22] This unit is 2.5 $\times$ the unit of Balls and Hoover.

Materials

Protein Substrates. Beef digestions were carried out on suspension of dried beef powder (Difco) in distilled water. For studying the effect of pH, the beef powder was suspended in 0.05 M citrate buffer.

Casein solutions of 6 per cent and 10 per cent concentration were prepared by dissolving Hammersten casein in water containing a volume (ml.) of N NaOH equal to the weight (gm.) of the casein. Citric acid and NaOH were then added in such ratio as to give the desired pH and in such amount that the solution, when diluted to final volume, was 0.05 M in respect to citrate. It was found that a pH of 5.4 was the lowest we could obtain without precipitation of the casein.

Commercial dried egg albumin (Merck) was used to prepare 5 per cent

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and 10 per cent solutions. These were buffered to the desired pH with sufficient phosphate or citrate buffer to give a 0.1 M concentration of buffer in the final solution. Crystalline bovine serum albumin (Armour) solutions of 5 per cent concentration were buffered in a like manner.

Gelatin of U.S.P. quality was used in preparing 10 per cent and 20 per cent solutions. These were buffered to the desired pH with sufficient phosphate, citrate, or acetate buffers to give a 0.03 M concentration of buffer in the final solution.

The buffered milk solution for the milk clotting was prepared according to the directions of Balls and Hoover.[22]

Papain Samples. N.F. Reference Papain and a commercial papain obtained from the American Ferment Co., as well as specially prepared papains, were used in this investigation. These latter samples were prepared from fresh latex in connection with studies of the effect of various factors on the activity and stability of papain. Detailed descriptions of their preparation will be reported later.

Papain Suspensions. In order to minimize any loss in activity of papain solutions,[24, 25] suspensions prepared as follows, unless specifically stated otherwise, were used: a suitable quantity of powdered (80–100 mesh) papain, accurately weighed, was suspended with the aid of glass beads in a measured volume of recently boiled, cold (less than 10°C.) distilled water. It is preferable to use the suspensions within three minutes after their preparation, and suspensions more than ten minutes old were discarded. Filtering these suspensions results in a loss of activity, probably by adsorption of some of the active enzyme by either the insoluble portion of the sample or by the filtering media.

Activation. Activation, where carried out, was accomplished by suspending 100 mg. of powdered papain, with the aid of glass beads, in 10 ml. of 0.1 N KCN solution at pH 7.0 and incubating at 40°C. for 30 minutes. These suspensions were then diluted to the required strength with cold, boiled, distilled water.

Experimental

The Effect of pH. From an examination of the literature, it appeared that the optimum pH for the digestion of casein[26–28] and egg albumin[29] by papain was near pH 7. Consequently, the trichloracetic acid precipitation method, employing casein as the substrate, was first used at a pH of 7.0–7.5. The first experiments in these laboratories, where the pH of the substrate could be accurately reproduced, were satisfactory. When the method was used in the field, however, where the pH of the substrate was adjusted with an indicator, the results were very erratic. This made it necessary to re-examine the effect of pH on the proteolytic activity of papain. FIGURE 1 gives the pH-activity curves of papain on the substrates, casein, egg albumin, and crystalline bovine serum albumin, as determined by the trichloracetic acid precipitation method. The curve for casein appears to be reaching a maximum at pH 5, whereas at pH 7.4 it has a steep slope, thereby explaining the above mentioned difficulties with this method. The curves

for egg albumin and crystalline bovine serum albumin are similar, showing maxima at pH 4 and minima at pH 6, the former even showing an apparent negative digestion at the latter pH. The formol titration method gave curves similar to those shown in FIGURE 1 for both casein and egg albumin. The curve for the latter is shown in FIGURE 2, where it can be seen that there are two maxima, one at pH 4, and the other at pH 7–8. The optimum in

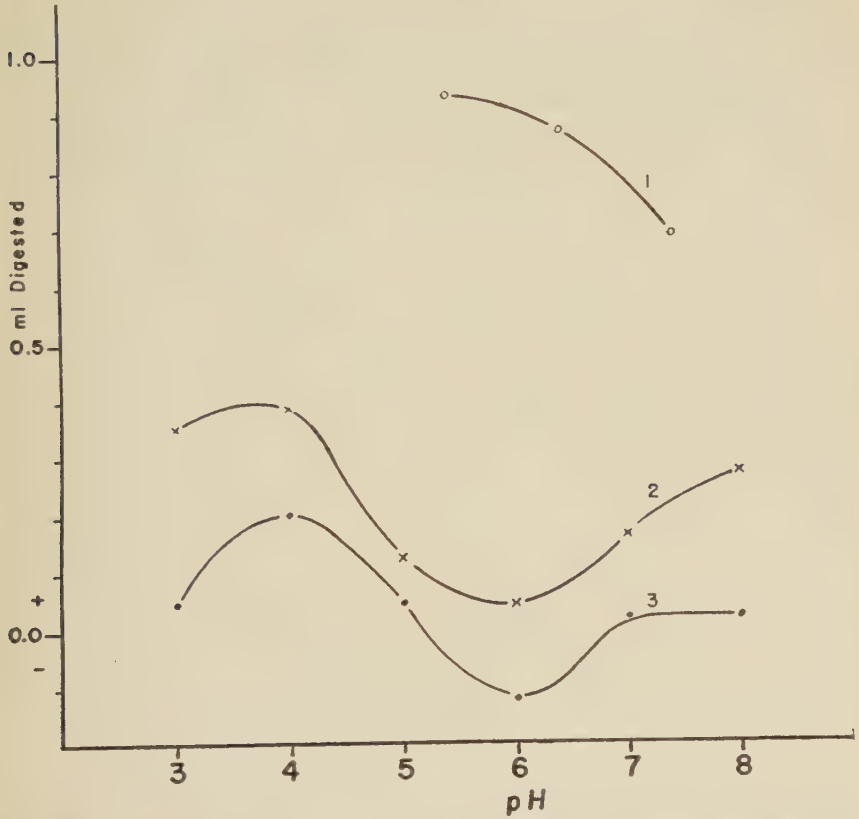

FIGURE 1. pH-activity curves. Curve 1 represents the digestion of 5 ml. of 0.05 M citrate buffered six per cent casein produced by 2 mg. papain in 30 minutes at 40°C. Curves 2 and 3 represent the digestion of 5 ml. of 0.1 M citrate-phosphate buffered five per cent crystalline bovine serum albumin and five per cent egg albumin, respectively, produced by 5 mg. papain in 30 minutes at 40°C. Commercial papain and the trichloracetic acid precipitation method were used.

this case is in the range of pH 7–8. Since the digestion in these experiments was of only 30 minutes duration, the pH activity curves in FIGURES 1 and 2 should be considered as representing the initial rate of digestion. These data do not agree with the published data, except in the case of the optimum at pH 7–8 for egg albumin[29] shown in FIGURE 2. Hoover and Kokes[19] found that although the initial rate of digestion of casein was greater at pH 7 than at pH 5 (practically three times as much digestion in 30 minutes), the digestion proceeded further at pH 5.

Of these three proteins, only casein was sensitive enough to the proteolytic action of papain to be of interest for use as a substrate. When it was used for the trichloracetic acid precipitation method, however, it precipitated as rather large, rubbery curds in the control tubes. The physical nature of this precipitate was such that it did not pack evenly, even after centrifuging at 1800 R.P.M. for 30 minutes. This introduced an additional variable of such magnitude that the variation in the volume of the controls alone amounted, in some cases, to as much as 20 per cent of the apparent digestion. For this reason, the method was of insufficient accuracy to be of further interest.

The pH-activity curves of the digestion of beef powder are shown in FIGURE 3. These data were obtained by the Maher and Wirth[5] method,

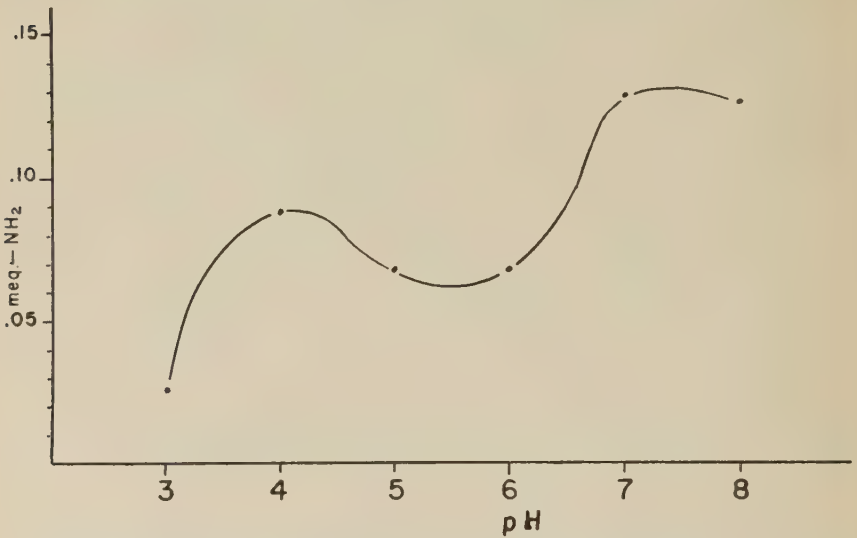

FIGURE 2. pH-activity curve of the digestion of 20 ml. of 0.1 M citrate-phosphate buffered ten per cent egg albumin produced by 40 mg. commercial papain in 30 minutes at 40°C. Formol titration was used to measure the extent of digestion.

using a fresh suspension of papain in boiled, cold water. Beef powder seems to swell with pH changes, the volume of the settled beef powder in the untreated controls, curve 1, steadily increasing from pH 4 to 8. On the other hand, the volume of undigested residue, curve 2, is practically constant from pH 5 to 8. This indicates an increase in digestion with increasing pH, curve 3. Since the residues in the control tubes are quite fluffy and slow in settling at the higher pH values and the residues in the digestion tubes are more closely packed and faster settling, however, it is not clear how much of this apparent increase in digestion is due to the actual proteolytic degradation of the beef powder. The pH of a slurry of the beef powder and water is between pH 5.7 and 6.0 and does not vary appreciably for different lots. Since this pH is on the flat portion of the digestion curve, as measured by the amount of undigested residue, the use of a buffer does not

seem to be necessary and may even be contraindicated, due to possible interference with the degree of settling of the beef powder in the controls.

The pH-activity curves of the digestion of gelatin, FIGURE 4, vary with the buffer used. The optimum pH for the digestion of gelatin is seen to be pH 5 with citrate buffer, curve 1, but is shifted to pH 4 with phosphate buffer, curve 3. Although no maximum was reached with acetate buffer,

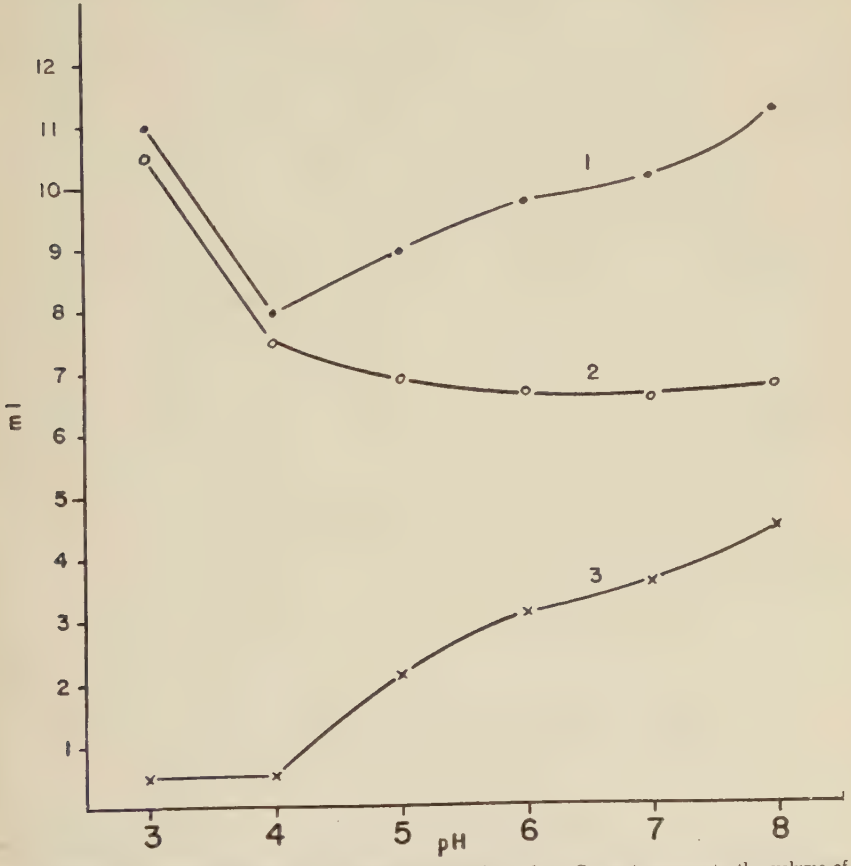

FIGURE 3. pH-activity curves of the digestion of beef powder. Curve 1 represents the volume of residue from 2 gm. of beef powder in controls containing no papain. Curve 2 represents the volume of undigested residue remaining after the digestion of 2 gm. of beef powder by 2 mg. of commercial papain at 70°C. for two hours. Curve 3 represents the amount of beef digested (Curve 2 values subtracted from Curve 1 values). 0.05 M citrate buffer was used.

curve 2, it is evident that the optimum is shifted still further, and probably is close to pH 3. These three buffers also affect the extent of the digestion as well as the location of the optimum pH, the digestion of gelatin by papain in the presence of phosphate buffer being only 80 per cent of that obtained in the presence of citrate or acetate buffers. Maschmann and Helmert[30] have also reported that the digestion of gelatin is greater with citrate buffers than with phosphate buffers. The reason for this change in the optimum

pH and the extent of digestion with a change in the buffer used is not known. It might possibly be due to the formation of protein-buffer complexes having differing properties. It would be interesting to see if this apparent change in activity with the buffer applies to other proteins. It is apparent that the digestion of gelatin by papain should be carried out using a substrate buffered at pH 5.0 with citrate buffer.

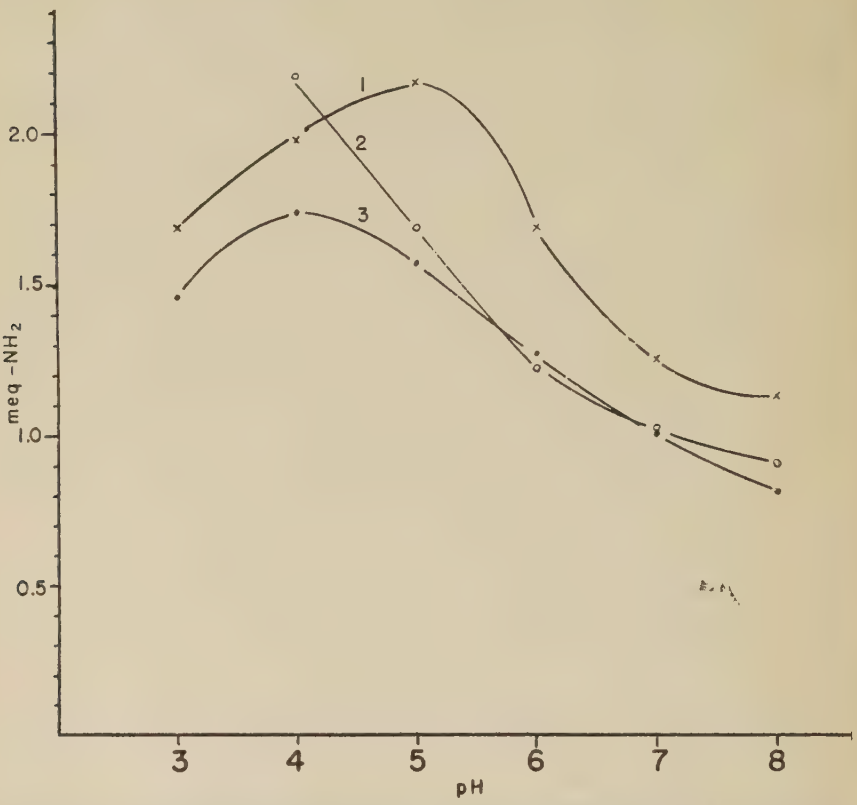

FIGURE 4. pH-activity curves of the digestion of 20 ml. of 0.03 M buffered ten per cent gelatin produced by 100 mg. of commercial papain in two hours at 40°C. Curve 1 is for citrate buffer, Curve 2 is for acetate buffer and Curve 3 is for phosphate buffer. Formol titration was used to measure the extent of digestion.

Effect of the Length of Time of Digestion. Since the digestion times (30 minutes for casein and two hours for gelatin) used in the investigation of the effects of pH were chosen arbitrarily, a study was made of the effect of varying the length of time of digestion. The formol titration method was used, the 6 per cent casein and 10 per cent gelatin substrates were buffered with citrate buffer at pH 5.45 and 5.0 respectively and digested with 100 mg. of papain. As can be seen from the time-activity curves, FIGURE 5, the digestion proceeds rapidly for the first hour and then slowly decreases. In both cases, it can be seen that variations in the digestion times of ± 2 or 3 minutes will result in much larger variations in the measured extent of

digestion at the 30-minute level than at the two-hour level of digestion. Since errors of timing were thereby minimized, a two-hour digestion period was used.

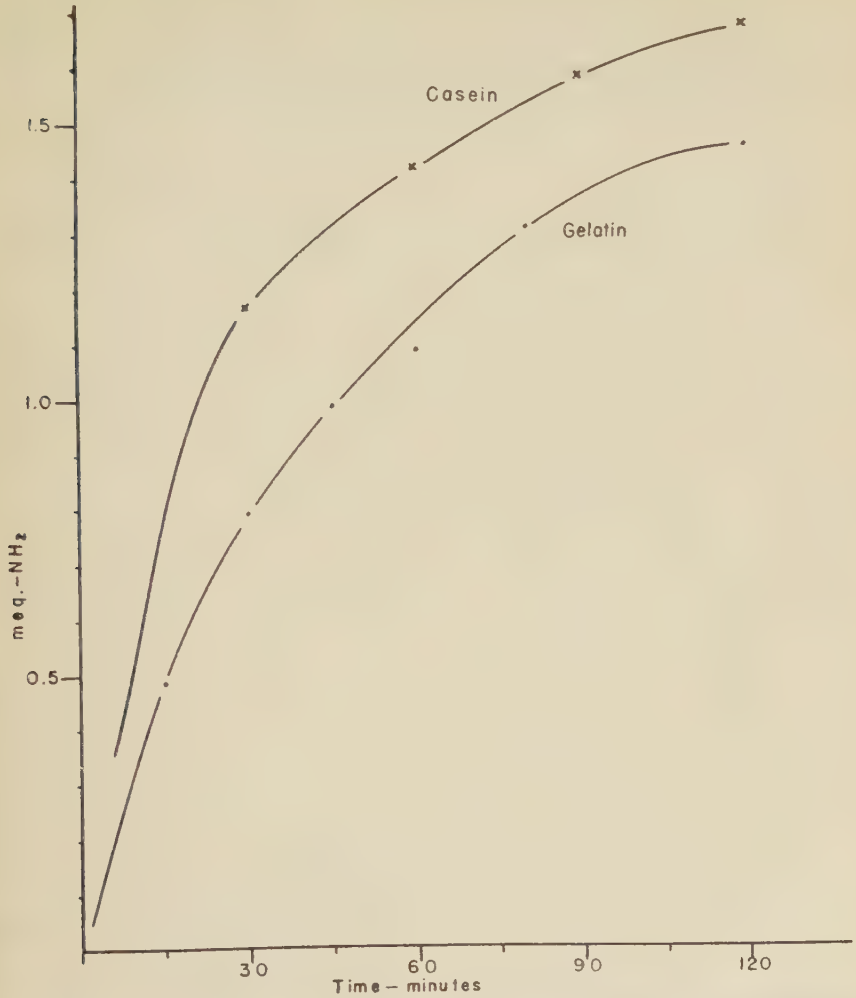

FIGURE 5. Time-activity curves for the digestion of 20 ml. of citrate buffered ten per cent gelatin (pH 5) and six per cent casein (pH 5.45) by 100 mg. of commercial papain at 40°C. Formol titration was used to measure the extent of digestion.

The Effect of Enzyme and Substrate Concentration. Since the usefulness of a method generally depends upon its sensitivity, the effect of varying the concentration of papain in the digestion mixtures was examined. The results of increased amounts of papain on the extent of the digestion of casein and gelatin are shown in FIGURES 6 and 7. These concentration activity curves are similar in slope to the time-activity curves shown in FIGURE 5 and

to that obtained by Maher and Wirth,[5] employing dried beef powder as the substrate.

These figures also show the effect of increasing the concentration of the substrate. In both instances, the slope of the curves at the higher substrate concentration is greater than at the lower concentration. This means that the sensitivity to changes in the enzyme concentration is increased by employing the stronger substrates. This increased sensitivity is offset, however, by the greater viscosity of the more concentrated substrates, making

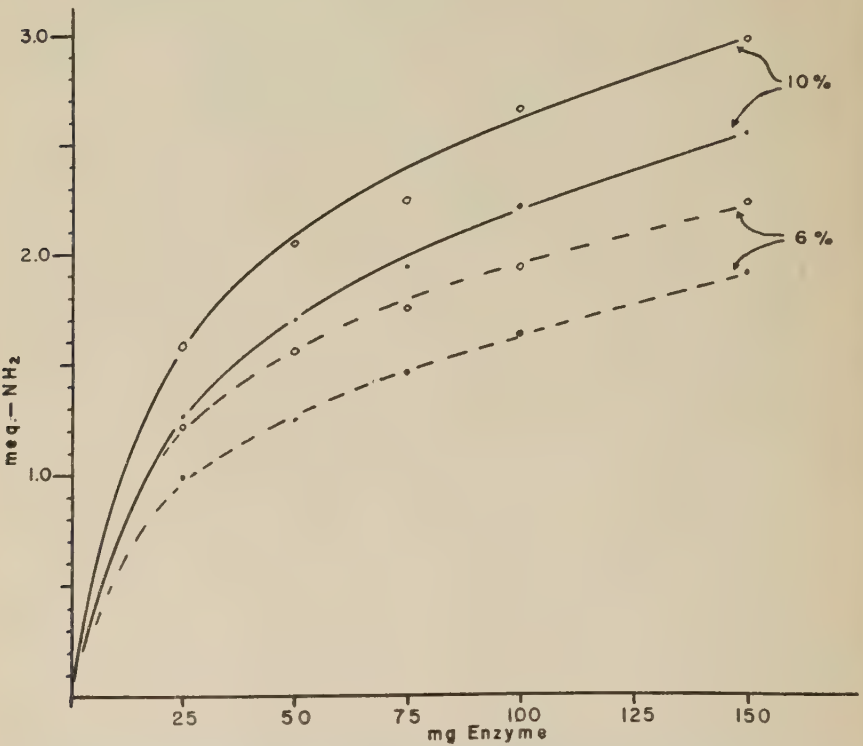

FIGURE 6. The effect of increasing the enzyme concentration on the digestion of 20 ml. of citrate buffered (pH 5.45) six per cent and ten per cent casein at 40°C. for two hours. Formol titration was used to measure the extent of digestion. Commercial papain ●—●, special papain ○—○.

it difficult to measure accurately the volume of substrate required for a digestion.

Since the casein solutions were even more viscous than the gelatin solutions, and since the results obtained on both substrates are practically identical, the use of citrate buffered (pH 5.0) 10 per cent gelatin solution as the substrate was adopted.

The Effect of Temperature of Digestion. All of the above digestions were carried out at an arbitrary temperature of 40°C. It seemed advisable from the work of Maher and Wirth[5] on beef powder to investigate the effects of temperature on the reaction. Buffered, pH 5.0, 10 per cent gelatin sub-

strates were digested by increasing quantities of three different papains at 40°, 55°, and 70°C. The results are given in FIGURE 8. It can be seen that the digestion proceeded slightly further at the higher temperatures. The commercial and the special papains showed the greatest activity at 55°C., whereas the N.F. Reference papain showed the greatest digestion at 70°C. Since the effect of the temperature was small in proportion to that of the other variables, and since most of the data reported in the literature were

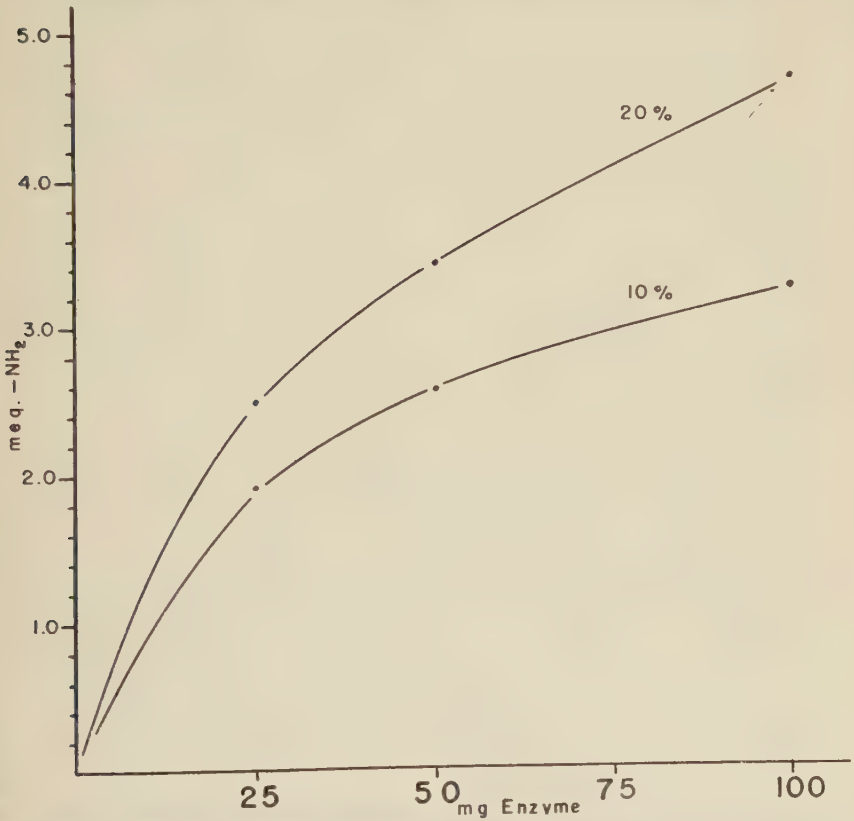

FIGURE 7. The effect of increasing the enzyme (commercial papain) concentration on the digestion of citrate buffered (pH 5) ten per cent and 20 per cent gelatin at 40°C. for two hours. Formol titration was used to measure the extent of digestion.

obtained from digestions carried out at 37–40°C., the advantages to be gained by being able to compare the present results with those of other investigators outweighed any to be gained by using a digestion temperature higher than 37°–40°C.

The Effect of Different Lots of Gelatin. Since gelatin is prepared by the hydrolysis of collagen, and cannot be assumed to be a homogenous protein, it seemed advisable to see if different lots of gelatin were digested to the same extent by papain. Three samples of U.S.P. quality gelatin from different

sources, were digested by each of two papain samples. The results, FIGURE 9, show that even gelatins of the same apparent quality respond differently to the proteolytic action of papain. Therefore, gelatin from the same lot was used for all subsequent studies.

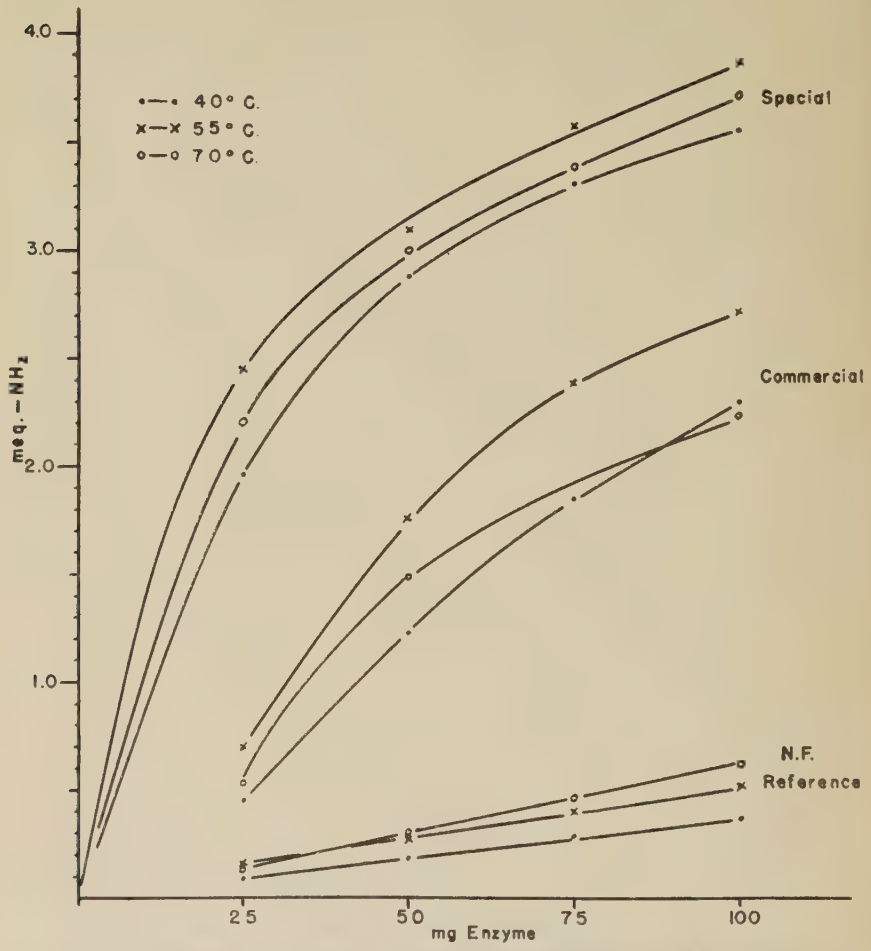

FIGURE 8. The effect of the temperature of digestion on the extent of digestion of 20 ml. of citrate buffered (pH 5) ten per cent gelatin produced by increasing amounts of three papains in two hours. Formol titration was used to measure the extent of digestion.

The Effect of the Age of Papain Solutions. Solutions of papain lose their milk-clotting activity upon standing,[24, 25] and it was observed that their gelatin digestion properties are also adversely affected. It seemed strange, therefore, that Maher and Wirth[5] recommended the use of 18-hour-old solutions of papain in their assay procedure. The N.F. procedure,[1] which also employs dried beef powder, specifies fresh suspensions of papain. In order to see if the age of the papain solution had any effect upon beef di-

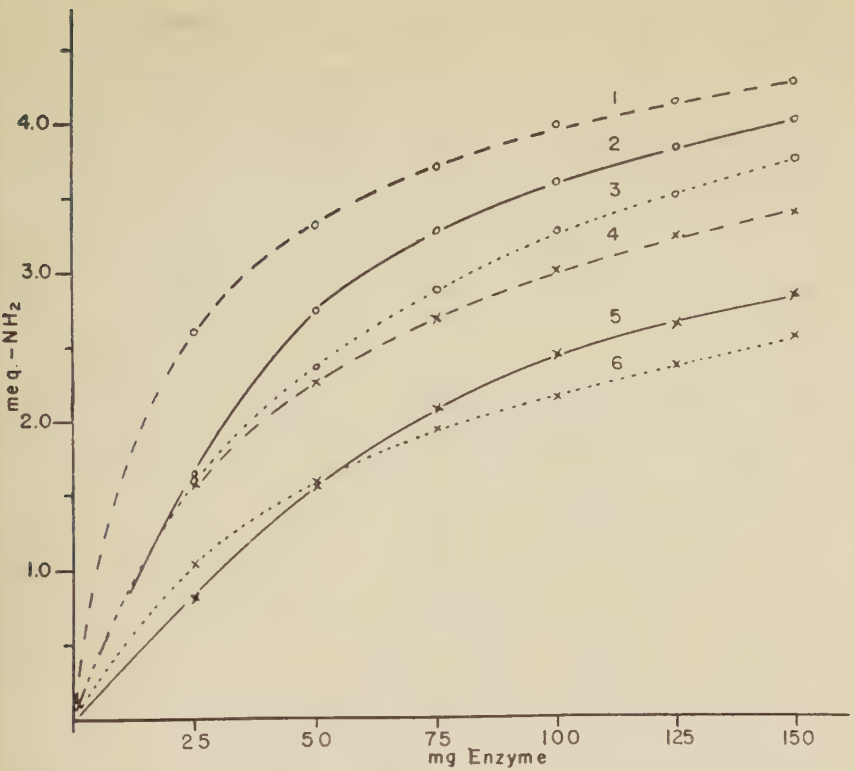

FIGURE 9. Variation in the extent of the digestion of three U.S.P. quality gelatins produced by two samples of papain at 40°C. for two hours. Curves 1, 2, and 3 compare the actions of the special papain on gelatins A, B, and C, respectively. Curves 4, 5, and 6 compare the actions of a commercial papain on the same gelatins. All three gelatins were used as ten per cent solutions buffered (citrate) at pH 5. Formol titration was used to measure the extent of digestion.

TABLE 1

THE EFFECT OF THE AGE OF PAPAIN SOLUTIONS ON THE DIGESTION OF DRIED BEEF POWDER

Papain sample	*Ml. beef digested*	
	Fresh suspension	*18-hour solution*
1	2.9	1.7
2	2.5	1.5
3	2.5	2.5
4	2.5	2.5
5	2.5	2.0
6	2.3	2.1

gestion, six samples of commercial papain were tested for their activity, using Maher and Wirth's[5] basic method. Both 18-hour-old solutions aged in the refrigerator, and fresh suspensions of the papain samples were employed. The results are given in TABLE 1. It is to be noted that the ac-

tivities of the 18-hour solutions were as great as the activities of the fresh suspensions in only two instances. As a result of these data, only fresh suspensions were used in the subsequent work done with this method.

Comparison of Methods. For comparative purposes, the activity of each of three samples of papain was tested by the following methods: the official N.F. method,[1] digesting 2 gm. beef powder with 100 mg. papain at 52°C. for 2 hours; the Maher and Wirth method[5] as modified above, digesting 2 gm. beef powder with 1 mg. papain at 70°C. for 2 hours; the centrifuge method, digesting 0.5 gm. beef powder with 0.5 mg. papain at 70°C. for 2 hours; the gelatin digestion method, digesting 20 ml. of citrate buffered (pH 5.0) 10 per cent gelatin solution with 100 mg. papain at 40°C. for 2 hours; and the Blau modification of the milk-clotting method of Balls and Hoover.[22] The three papain samples were a commercial papain, a specially prepared papain, and the N.F. Reference papain. The results are summarized in TABLE 2 together with relative activities. It can be seen that the results obtained by the three beef methods agree very well. The other

TABLE 2

THE ACTIVITY OF THREE PAPAINS AS DETERMINED BY FIVE METHODS OF ASSAY*

Papain sample	*Beef methods*						*Milk clotting*		*Gelatin digestion*	
	N.F.		*Maher & Wirth*		*Centrifuge*		*UA/gm.*	%	*mEq.-NH_2*	%
	ml.	%†	*ml.*	%	*ml.*	%				
N.F. Reference	5.3	87	3.4	79	0.38	76	10	2	0.73	19
Commercial	5.5	91	3.8	88	0.38	76	110	22	3.05	79
Special	6.0	100	4.3	100	0.50	100	448	100	3.89	100

* Average of duplicate determinations.
† The percentage values are in terms of the special papain activity.

two methods, however, give results that do not agree either with those of the beef methods or with each other. The N.F. Reference papain is practically inactive by the milk-clotting method and only slightly more active by the gelatin digestion method.

Gottschall[31] has noted that papain which is inactive by the "usual tests" may be very active in the digestion of meat. He explains this as being due to the activation of the papain by sulfhydryl groups liberated during the proteolysis. If this is the case, the activation of the papain prior to testing by the milk-clotting and gelatin-digestion methods should produce results comparable with those obtained by the beef digestion methods. TABLE 3 gives the data obtained by the activation (HCN) of the papain prior to testing. On comparing the percentage results with those in TABLE 2, it is seen that the gelatin method, when the papain is activated, gives results that are practically the same as those obtained by the beef methods. The milk-clotting method, however, although the N.F. Reference and commercial papains show higher activities on activation, still does not produce results comparable with the others.

It has been stated that there is no correlation between the milk-clotting and proteolytic activities of papain.[32] Some[5] have proposed that the milk-clotting activity is due to a different enzyme. These statements are not in agreement with the high milk-clotting activity for both of the crystalline proteolytic enzymes, papain[33] and chymopapain,[34] that have been isolated from papaya latex. Since both of these crystalline enzymes have high proteolytic and milk-clotting activity, it seemed that the latter property was only one manifestation of the former. If this were true, however, there should be some way to correlate the two.

Balls and Hoover[22] have also shown that the time required for clotting is a straight-line function of the enzyme concentration. On the other hand, the digestion is not a straight-line function of the enzyme concentration for gelatin (FIGURE 7), casein (FIGURE 6), or beef[5] digestion. The present data show, however, that in these latter cases the response is a straight-line function of the logarithm of the enzyme concentration. To illustrate this, some of the data for FIGURES 6 and 7 have been replotted in FIGURE 10, as response against the logarithm of the enzyme concentration. Maher and Wirth's beef digestion data (reference 5, curve *c*, Figure III, page 63) have

TABLE 3
THE ACTIVITY OF THREE PAPAINS ACTIVATED WITH HCN PRIOR TO PROTEOLYSIS

Papain sample	*Milk clotting*		*Gelatin digestion*	
	UA/gm.	%	*mEq.-NH_2*	%
N.F. Reference	108	25	2.98	77
Commercial	188	41	3.46	89
Special	458	100	3.88	100

also been replotted as response versus the logarithm of the enzyme concentration and found to be a straight line. Hoover and Kokes[19] have previously noted that for their data, and others,[27] the extent of the digestion of casein was a straight-line function of the logarithm of the time of digestion for 1 to 100 hours and beyond. The present time-digestion data given in FIGURE 5, when replotted against the logarithm of the time, also show this linear relation. In contrast to Hoover and Kokes, it was found that this linearity holds from 30 and 15 minutes on for casein and gelatin, respectively. In the other data in the literature for papain, as well as for other proteolytic enzymes, there is also a linear relationship between the response and the logarithm of the enzyme concentration and the time.

Regardless of the fundamental significance of the above linear relations, they can be used as a basis for evaluating the different methods of estimating the proteolytic activity of papain. It is here shown that the extent of digestion by both the gelatin and the beef methods is a linear function of the logarithm of the enzyme concentration. If the two methods are measuring the same activity, therefore, their responses should vary simultaneously, and a plot of ml. of beef digested versus milliequivalents of $-NH_2$ liberated from gelatin for different papain samples should be a

straight line. Upon plotting the data in TABLES 2 and 3 in this manner, the beef methods appeared to correlate with the gelatin method provided the papain was activated prior to testing by the latter method.

The milk-clotting method, as stated before,[22] measures clotting time that is a linear function of the enzyme concentration. The activity of a sample of papain is very easily calculated as units of activity per unit weight of sample. If the milk-clotting method, therefore, is measuring the same action of papain as the gelatin and beef digestion methods, the response by the latter methods will be a linear function of the logarithm of the units of

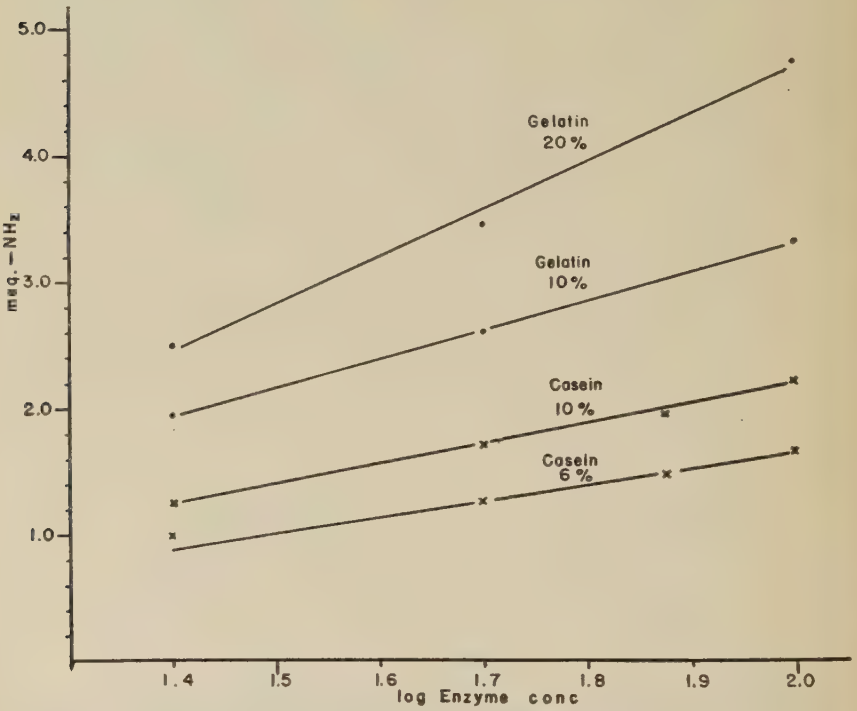

FIGURE 10. Illustration of the linear relationship between the response and the logarithm of the enzyme concentration. This is some of the data shown in FIGURES 6 and 7. Commercial papain was used and the extent of the digestion was measured by formol titration.

activity per unit weight measured by the former method. The data in TABLES 2 and 3 were plotted, ml. beef digested *versus* logarithm of UA/gm., and mEq.-NH_2 liberated versus logarithm UA/gm. It was found that, whereas the milk and gelatin results gave apparent straight lines for both the native and activated papain data, the beef data gave a straight line with only the milk-clotting data obtained with the activated papains.

The data from the above set of experiments indicate that the beef-digestion, gelatin-digestion and milk-clotting methods measure the same property of papain. They also indicate that papain is activated during digestion when beef is used as the protein substrate, as has been noted before by

Gottschall.[31] There are no data available at this time which indicate whether the reversibly inactive enzyme is activated *in vivo*. Preliminary *in vitro* tests in which samples of papain were incubated with canine gastric juices failed to show any activation of the enzyme.

Since all three methods appear to give comparable results, the choice of a method to use for the assay of papain depends upon their relative merits *per se*. All three methods require a minimum of special apparatus and technique. Although the milk-clotting method employs a special technique, Blau's modification, given here, is very simple. There is no great choice between the three substrates as to their uniformity, since all vary to a greater or less extent. There is a large difference, however, in the sensitivity of the methods. Both the beef and the gelatin methods give results that are linear functions of the logarithm of the activity of the papain being tested. This means that two papains having a ratio of activity of 1:2 will not give responses of 1:2, but rather responses of log 1:log 2 or 1:1.3. On the other hand, the milk-clotting method gives results that are in direct proportion to the activities of the samples of papain being tested.

All things considered, the milk-clotting method is probably the method of choice for measuring the activity of papain. It is not only faster and more sensitive than the other two methods, but it can also be used to measure the proportions of active and reversibly inactive enzyme present in the sample.

Summary

A study of assay methods for papain and the factors affecting them has been made.

The optimum reaction of the digestion of casein is about pH 5 and is pH 3.5–4.0 for the digestion of crystalline bovine serum albumin. Commercial dried egg albumin shows maxima at both pH 4 and pH 7–8, with the optimum depending upon the method employed to measure the extent of digestion. The optimum pH for the digestion of gelatin varies with the buffer system employed, being pH 5 for citrate buffer, pH 4 for phosphate buffer, and approaching pH 3 for acetate buffer. Beef powder does not exhibit any clear maximum, the digestion being practically constant from pH 5 to 8.

It has been shown that the extent of digestion is a linear function of the logarithm of the enzyme concentration for the substrates beef powder, gelatin, and casein. The extent of digestion has also been shown to be a linear function of the logarithm of the time, as has been noted before by Hoover and Kokes. These linear relationships have been used as a basis for comparing the beef-digestion, gelatin-digestion, and milk-clotting methods of measuring the activity of papain. The beef digestion reflects the total enzyme content of papain, both the active and reversibly inactive forms, whereas neither the milk-clotting nor gelatin methods measure the reversibly inactive enzyme unless the papain is activated prior to testing.

It has been shown that results obtained by the three methods appear to be correlated and, therefore, are measures of the same property of papain.

The modified milk-clotting method, because of its simplicity and sensitivity, is the method of choice for the routine assay of papain.

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CORRELATION OF THE RESULTS OBTAINED BY BEEF-DIGESTION, GELATIN-DIGESTION, AND MILK-CLOTTING METHODS OF MEASURING THE PROTEOLYTIC ACTIVITY OF PAPAIN

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In a recent report[1] from these laboratories dealing with various aspects of measuring the proteolytic activity of papain, data were given that seemed to show that, under certain conditions, the results obtained by beef digestion, gelatin digestion, and milk clotting were correlated. Since those data were quite meager, however, additional investigations have been made of this point.

Sixty different samples of papain were examined. All except two of these had been prepared from fresh latex, in connection with other studies concerning the effect of various factors on the activity and stability of papain.[2–4] National Formulary Reference papain* and a commercial papain, tested before,[1] completed the series of samples.

Methods of Assay. Three methods were employed to measure the activity of the papain samples. Milk clotting was determined by the Blau modification[1] of the Balls and Hoover method,[5] employing 25 ml. of a standard milk solution at 40°C. Beef digestion was measured, utilizing the method of Maher and Wirth,[6] modified by the use of fresh suspensions of papain.[1] Two grams of dried beef powder were digested with 1 mg. of papain for 2 hours at 70°C. Gelatin digestion was followed by formol titration.[1] Twenty ml. of 0.03 M citrate buffered, pH 5.0, 10 per cent gelatin solution was digested with 100 mg. of papain for 2 hours at 40°C.

The milk-clotting and gelatin-digestion activities were measured both with and without prior activation of the papain, whereas the beef digestion activities were only measured without prior activation. All determinations were made in duplicate, with the exception of the gelatin digestion set employing activated papain. In this latter case, there was not enough of some of the samples to run the assays in duplicate and still have sufficient material remaining to complete the studies for which they were prepared.

Activation. The papain samples were activated by incubating 100 mg. of papain with 10 ml. of 0.1 N KCN solution, adjusted to pH 7.0 with HCl, at 40°C. for 30 minutes.

Results. The results of these activity determinations are given in TABLE 1. The responses elicited by the various samples of papain are reported as "per cent," the average response produced by the N.F. Reference papain taken as 100. In the case of milk clotting, the results are given as "per cent" of the logarithm of the units of activity per gram, so that they can be directly compared with the beef and gelatin values.[1] It should be pointed out that these "per cent" figures do not refer to the activity of the samples, but only to their response. This transformation to per cent response is desirable in

* Obtainable from the Chairman of the Committee on National Formulary, American Pharmaceutical Association, Washington, D. C.

order to simplify the arithmetic involved in the statistical treatment of the data.

The average difference between runs and the standard error of the average difference are given in TABLE 1 for all the methods except gelatin digestion, activated, where only one run was made. It can be seen that there is a significantly greater average difference and standard error for the beef values than for the others. This greater variability of the results obtained by beef digestion is more clearly illustrated in TABLE 2. Although there is a practically perfect correlation between runs for the milk and gelatin data ($r = 0.999$, $r = 0.999$, and $r = 0.997$), the correlation between runs for the beef results was considerably less ($r = 0.797$). This shows that there is little or negligible inherent variation in the milk and gelatin methods, although there is a considerable variation inherent in the beef method. This variation in the response obtained by the beef method can be expected to influence adversely the magnitude of any correlation between this method and the others under consideration.

The correlation coefficients calculated from the data in TABLE 1 for the six pairs of data (Milk × Gelatin; Milk, Activated × Gelatin, Activated; Milk, Activated × Beef; Gelatin, Activated × Beef; Milk × Beef; and Gelatin × Beef) are given in TABLE 3. There is a very good correlation between the results obtained by the milk and gelatin methods. The correlation coefficients ($r = 0.9$ in both cases) indicate a very good agreement between the two methods. This lends very strong support to our belief that these two methods measure the same combination of properties of papain. Although the correlations between the beef data and both the milk-activated and gelatin-activated data are considerably less, the coefficients, $r = 0.53$ and $r = 0.55$, respectively, are nevertheless quite significant [$p < 0.001$[7]].

Because of the great variations inherent in the beef method, no high correlation could reasonably be expected between the results with this method and those of the more reproducible techniques. In order to test this assumption, other beef-digestion values obtained in duplicate for the 60 samples were similarly analyzed. The correlation coefficient between runs of this additional set of data was found to be $r = 0.75$, which is practically the same as that obtained from the first beef data. Upon comparing the averages of the two sets of beef results, a correlation coefficient of $r = 0.41$ was found, which is even less than those found for the beef- and milk-activated, and beef- and gelatin-activated results.

The correlations for the beef data with both the milk-clotting and gelatin-digestion unactivated results are even less, $r = 0.32$ ($p = 0.01$) in both cases. Since all the samples of papain contained some of their enzymes in the active state, but in varying proportions to their total enzyme content, some correlation is to be expected, but less than that obtained upon activation.

A clearer picture may be had from plotting the data in TABLE 1. The relations between the gelatin and milk results are graphically illustrated for the unactivated series in FIGURE 1 and for the activated series in FIGURE 2. It is easy to see the very good agreement between these methods.

FIGURE 3 shows the relation between the beef and milk clotting, activated, and FIGURE 4 illustrates the relation between the two sets of beef results.

TABLE 1

RESPONSE VALUES IN PER CENT (N.F. REFERENCE PAPAIN = 100) OF 60 PAPAIN SAMPLES OBTAINED BY BEEF-DIGESTION, MILK-CLOTTING, AND GELATIN-DIGESTION METHODS OF MEASURING PROTEOLYTIC ACTIVITY

Sample	*Beef Digestion*		*Milk Clotting (Unactivated)*		*Milk Clotting (Activated)*		*Gelatin Digestion (Unactivated)*		*Gelatin Digestion (Activated)*
	1	2	1	2	1	2	1	2	
21Aa	126	121	210	211	110	111	438	429	117
21Ab	124	109	169	168	108	108	238	238	112
24A	106	112	179	180	104	103	251	256	112
21Ba	85	79	169	166	100	100	391	396	96
24B	109	79	179	176	93	94	360	364	93
21Da	79	85	196	197	92	92	329	333	101
24D	115	126	219	216	98	98	333	333	115
28Ba	132	132	208	208	117	117	416	420	121
28Bb	132	132	151	150	105	105	169	164	109
29A	115	115	167	164	97	96	233	224	98
33A	132	138	191	190	107	107	333	329	114
34Da	138	138	204	202	107	108	396	396	118
b	124	138	207	207	108	108	411	404	118
Ea	118	124	123	121	100	100	160	156	109
b	129	124	143	143	103	103	196	200	109
Fa	132	132	247	247	120	120	584	589	126
b	124	124	221	220	105	105	447	456	115
Ga	91	109	167	167	87	87	204	204	87
b	85	97	187	187	90	88	282	282	90
35A	112	112	216	215	108	108	338	333	112
41A	132	138	207	207	112	112	442	442	124
46Aa	132	126	212	211	114	114	487	493	121
b	132	121	207	207	111	111	447	444	120
d	121	121	189	190	106	105	369	373	116
e	115	115	207	207	108	108	407	416	118
f	118	124	185	184	106	105	256	260	114
Ba	118	118	213	213	108	108	378	378	121
b	106	118	200	200	108	108	324	320	117
d	115	121	227	227	114	115	520	520	132
e	129	112	229	229	117	117	538	538	121
f	124	106	176	174	103	105	260	260	118
Ca	124	124	211	210	111	111	396	396	121
b	121	121	209	207	111	110	351	360	121
d	115	109	234	234	117	117	602	602	128
e	109	109	226	225	114	114	538	538	125
f	106	106	180	180	106	106	260	260	115
Da	115	109	245	243	119	120	602	600	124
b	126	115	259	259	130	129	720	716	136
d	112	112	256	256	125	125	716	711	132
e	124	124	259	257	123	124	707	707	135
f	103	109	240	239	111	111	538	536	122
Ea	106	112	217	216	98	98	218	216	96
b	103	97	167	167	90	90	187	193	93
d	126	115	234	234	115	114	527	522	123
e	118	100	234	234	115	115	522	529	121
f	115	91	161	163	100	101	204	202	89
Fa	88	79	179	179	96	96	224	224	93
d	115	121	238	239	118	118	529	538	124
e	115	103	234	235	116	116	549	542	119
f	97	103	160	160	99	99	200	200	109

TABLE 1—*Continued*

Sample	*Beef Digestion*		*Milk Clotting (Unactivated)*		*Milk Clotting (Activated)*		*Gelatin Digestion (Unactivated)*		*Gelatin Digestion (Activated)*
	1	2	1	2	1	2	1	2	
Ga	97	97	215	216	110	111	498	493	124
b	94	94	215	216	114	112	507	502	122
d	94	106	229	228	116	115	569	560	121
e	97	109	206	206	106	108	411	411	118
f	109	115	159	159	110	109	356	356	114
62A	121	121	205	204	110	111	396	402	115
62B	138	153	263	261	130	129	724	720	124
Narran B	124	118	200	201	111	112	524	520	117
N.F. Ref.	103	97	102	98	100	100	100	100	100
"Bilsulfite"	138	138	259	259	131	131	776	780	135
Av. diff. between runs	6.77		0.90		0.47		3.50		
St. error of av. diff.	0.87		0.13		0.08		0.39		

TABLE 2

CORRELATION COEFFICIENTS BETWEEN RUNS OF THE BEEF-DIGESTION, GELATIN-DIGESTION, AND MILK-CLOTTING METHODS OF MEASURING THE PROTEOLYTIC ACTIVITY OF PAPAIN

Method	*r*
Milk Clotting, not activated	0.999
Gelatin Digestion, not activated	0.999
Milk Clotting, activated	0.997
Beef Digestion	0.797

TABLE 3

CORRELATION COEFFICIENTS BETWEEN METHODS

Method	*r*
Milk Clotting × Gelatin Digestion	0.90
Milk Clotting, activated × Gelatin Digestion, activated	0.90
Beef Digestion × Gelatin Digestion, activated	0.55
Beef Digestion × Milk Clotting, activated	0.53
Beef Digestion × Gelatin Digestion	0.32
Beef Digestion × Milk Clotting	0.32

It is apparent that the beef- *versus* milk-clotting results are in better agreement than the two sets of beef data. The beef results are plotted *versus* the milk-clotting, not activated, results in FIGURE 5. The poor correlation between these methods is readily apparent.

Discussion. Since the response obtained by the gelatin-digestion method of testing papain is dependent upon the number of amino groups liberated by the enzyme, it is apparent that this method measures the proteolytic activity of papain. The milk-clotting activity of papain has been considered as being different from the proteolytic activity. As a consequence of the

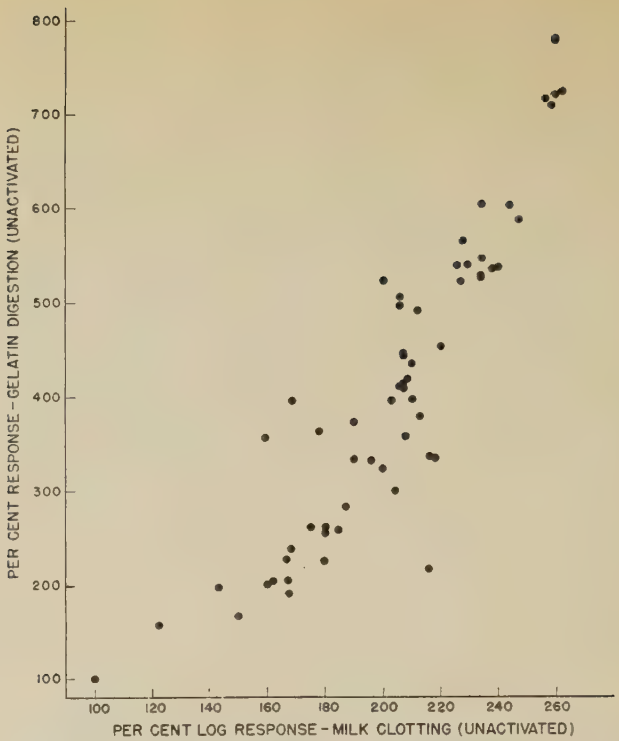

FIGURE 1. Graphic illustration of the correlation between the results obtained by gelatin-digestion and milk-clotting methods of measuring the proteolytic activity of papain. The enzyme preparations were not activated prior to testing. $r = 0.90$.

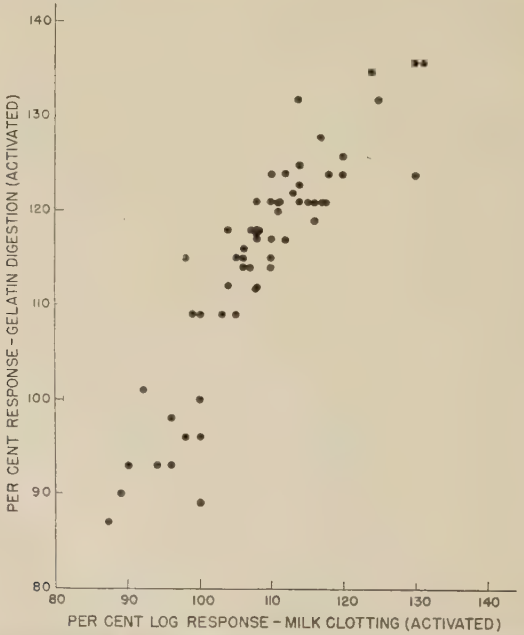

FIGURE 2. Graphic illustration of the correlation of the results obtained by gelatin-digestion and milk-clotting methods of measuring the proteolytic activity of HCN activated papain. $r = 0.90$.

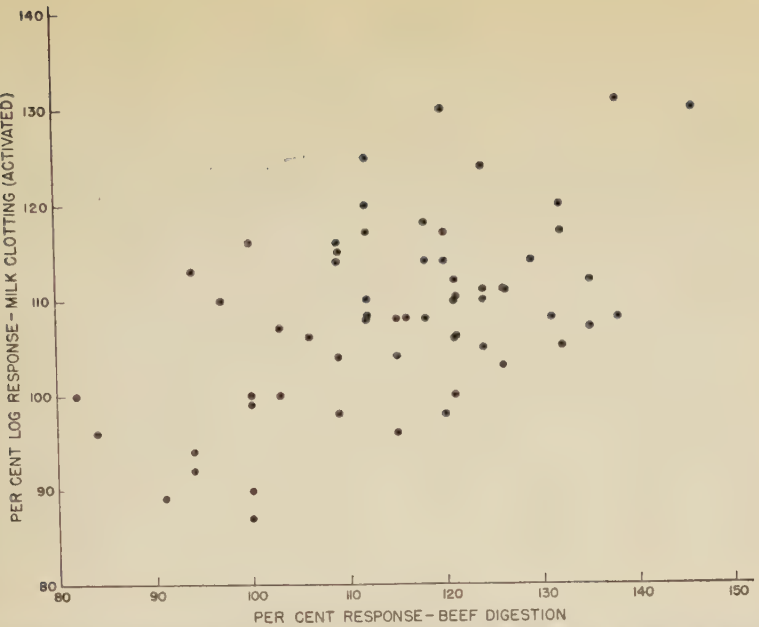

FIGURE 3. Graphic illustration of the poor correlation of the results obtained by beef-digestion and milk-clotting methods of measuring the proteolytic activity of papain. The enzymes were activated with HCN prior to testing by the milk-clotting method but were not activated prior to testing by the beef-digestion method. $r = 0.53$.

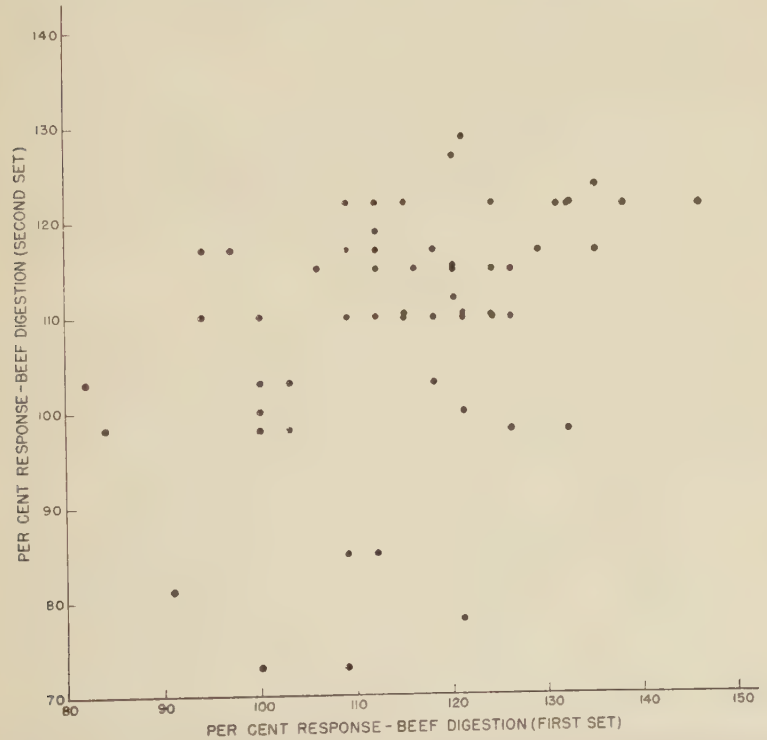

FIGURE 4. Graphic illustration of the low degree of correlation between two sets of results obtained by the beef-digestion method. The same unactivated papain samples were used in each case. $r = 0.41$.

data presented here, however, it is evident that the two methods are measuring the same property of papain.

The results obtained by beef digestion, on the other hand, correlate with the results obtained by the other two methods only after activation of the enzymes. Further support for this view was obtained as a consequence of another study.[8] Two papain preparations were employed and their ac-

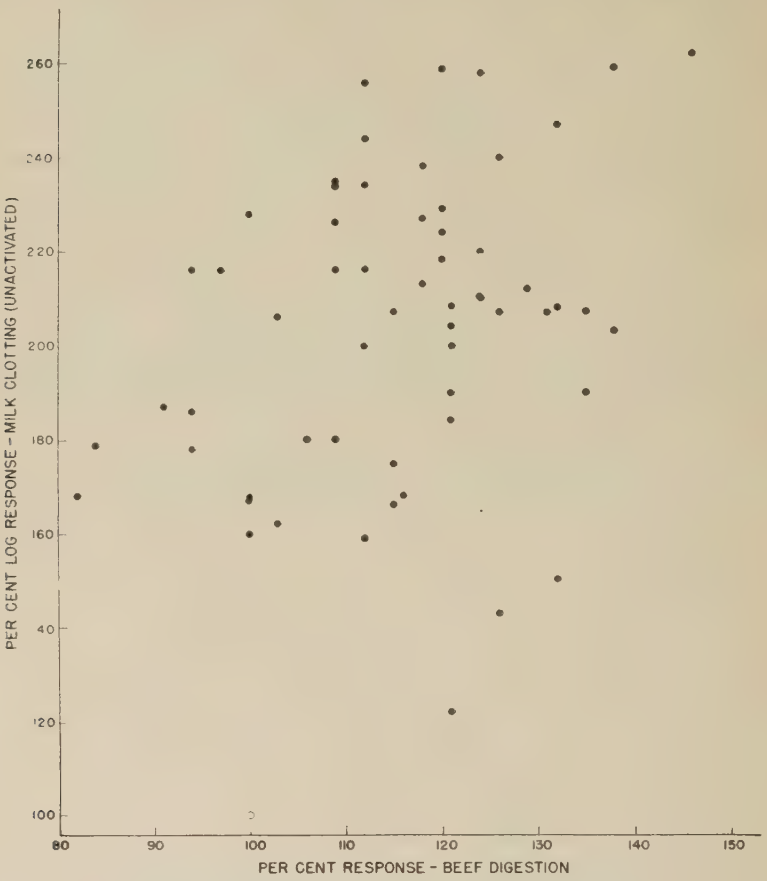

FIGURE 5. Graphic illustration of the low degree of correlation of the results obtained by beef-digestion and milk-clotting methods of measuring proteolytic activity. Unactivated papain samples were used. $r = 0.32$.

tivities were determined by milk clotting both with and without activation, by egg digestion,[9] and by beef digestion (not reported). The ratios of the activities of these two papains were found to be: 1:75 by milk clotting, unactivated; 1:3.5 by milk clotting, activated; 1:1.5 by egg digestion; and 1:1.4 by beef digestion. It is readily apparent that the activated milk-clotting results are of the same magnitude as the other two. These correlations are not as striking as those obtained for the milk-clotting and gelatin-digestion data. It must be realized, however, that the tremendous variability of the results obtained by the beef method preclude any greater

correlation. This is especially true when the low degree of correlation between different sets of beef data is considered.

The three methods, beef digestion, gelatin digestion with activation, and milk clotting with activation, give comparable results, and are apparently measuring the same property of papain. It is thus evident that a choice between them will be governed by the relative merits of each method, *per se*. The relatively great variability of the results obtained by the beef method almost preclude it *a priori* from further consideration. An objection to the gelatin method is brought out in a previous paper,[1] which showed that different lots of gelatin of the same apparent quality are not digested to the same extent by papain. On the other hand, the milk-clotting method is faster and more reproducible than the other two methods. The sensitivity of the milk-clotting method is also greater, since the response obtained by this method is proportional to the activity of the sample rather than to the logarithm of the activity, as is the case with both the gelatin-digestion and beef-digestion methods.[1, 5] In view of these superior merits, the milk-clotting method is the method of choice.

Summary

The results obtained by beef-digestion, gelatin-digestion, and milk-clotting methods for measuring the proteolytic activity of papain have been compared. Sixty samples of papain were tested. It was found that the results obtained by both gelatin-digestion and milk-clotting methods are highly correlated, and that the two methods, therefore, measure the same property of papain.

Beef-digestion results agree with the results obtained by the gelatin-digestion and milk-clotting methods only when the papain is activated prior to testing by the latter methods. This agreement is adversely influenced by the inherent variability in the beef method.

The speed, reproducibility, and sensitivity of the milk-clotting method make it the method of choice for measuring the activity of papain.

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A NEW METHOD FOR THE ASSAY OF PAPAIN

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Papain is described by the National Formulary[1] as "the dried and purified latex of the fruit of *Carica Papaya*, Linne (Fam. *Caricaceae*). It possesses a digestive activity not less than that of the Reference Papain."

Papain has long been known to have proteolytic properties. From time immemorial, the Indians are said to have used the fresh leaves of the papaya plant to tenderize meat. Some of the papain imported into this country is used for this purpose.[2] A greater amount, however, is used as an aid to protein digestion. It has also found use in medicine and in surgery in preventing postoperative peritoneal adhesions,[3,4] in the treatment of sloughing wounds,[5] for the dissolution of the eschar of burns,[6] in the treatment of chronic purulent otitis media,[7,8] *etc.*

Although a highly purified crystalline papain has been prepared by Balls and Lineweaver,[9] very little is known regarding the physical constants and the true chemical nature of the enzyme molecule. It has been demonstrated that beef serum, pseudoglobulin, and diphtheria antitoxin are split by papain into fragments of one-half and one-quarter of the original molecular weight.[10] In addition to its proteolytic power, papain possesses some protein-synthesizing power.[11] Papain, as well as trypsin, also liberates histamine from living tissues.[12]

There is known to be considerable variation between the proteolytic activity of different lots of papain. This variation may be attributed to one or more factors. In the first place, the collection and drying of the latex are done in many different localities, often with rough handling and very little care. Some commercial papain has been found to be adulterated.[13] Certain compounds containing the sulfhydryl or cyanide radical have the power of activating papain.[14] Gottschall[15] has observed that materials such as beef muscle, liver, and new beer will activate papain spontaneously. In addition to the above factors, a method of assay has not been available which would furnish uniform reproducible results in different laboratories, since some substrates require activation of the papain to produce total potential activity, while other substrates do not require activation.

Assay Methods. The general methods of assay of papain may be classified according to the substrates used: beef, casein, gelatin, hemoglobin, milk, and egg albumin.

The use of lean, ground beef as a substrate is one of the oldest methods reported[16] in the literature. The National Formulary[1] has adopted this as the official assay procedure, using dried beef powder. The method consists of comparison of the volume of the residues after a two-hour digestion at 52°C. of aqueous beef powder substrates which contain equal amounts of unknown and Reference papain. The official assay, however, may be insufficiently selective or sensitive to changes in the amounts of papain used to permit quantitation of results obtained.[17]

Proteolytic activity of papain has been determined by the digestion of

casein and subsequent polarization,[18] digestion of casein, and titration with alcoholic potassium hydroxide using a control without papain,[19] or by digestion of casein and formol titration.[20]

The digestion of gelatin, as measured by polarization, also affords a means of determining activity of papain.[21] Another method has been reported in which the activity is based on the extent of digestion of the gelatin on a strip of photographic film, using citrate solutions as activators.[22] The formol titration with gelatin substrate has also been used.[23] In a recent study,[17] the activity was expressed in the number of "milligram-hours" of papain necessary to liquefy a specified quantity of gelatin solution. This is based on the fact that the product of milligrams of papain times the hours of hydrolysis was found to be a constant in the liquefaction of a specified quantity of gelatin solution. No correlation was found, however, between the beef-digestion activity of papain and its ability to liquefy gelatin solutions.

The use of hemoglobin[24] as a single, pure protein substrate serves as a means of estimation of proteolytic activity of papain if the enzyme is properly activated with cyanide.

The utilization of the milk-coagulation property of papain, originally shown by Balls and Hoover[25] as a determinant of activity, is the most widely quoted method in the literature. Gottschall[15] has pointed out that milk clotting is not a measure of the total proteolytic activity unless the papain is first activated by cyanide, since the milk will clot before the protein is digested to any great extent. Recent work by Hinkel *et al.*,[26] however, indicates good correlation between the beef- and milk-clotting methods when activated papain is used in the latter method.

There have been very few reports of the use of egg albumin for determining the proteolytic activity of papain. In 1912, Rippetoe[27] advocated the use of coagulated egg albumin, using a six-hour digestion at 52°C. with 0.1–0.2 gm. of papain in an alkaline media. Heyl, Caryl, and Staley[28] in 1914 reported a method using an unboiled standardized solution of egg albumin and determined the activity by the percentage of protein rendered noncoagulable after a fifteen-minute treatment in a bath at 80°C.

The fact that papain will digest egg albumin is well known, however. Svedberg and Erikson[29] and Annets[30] have analyzed the products of the action of papain on egg albumin. Other references to the proteolysis of egg albumin by papain include the work of Greenberg and Winnick,[31] Calvery,[32] and Willstätter, Grossman, and Ambros.[33]

The comparative scarcity of reports of proteolysis of egg albumin as a measure of papain activity indicated this approach had not been explored as thoroughly as some of the others and that the use of egg albumin as a substrate warranted further study.

Development of Egg Albumin Assay. The initial procedure followed in this study was that outlined by the National Formulary[1] for the assay of papain, with the exception that dried egg albumin was used in place of the dried beef powder. There were only insignificant differences in digestion with varying amounts of papain.

It was then decided to substitute freshly coagulated egg albumin for the dried egg albumin. The egg albumin was prepared in the manner described in the official pepsin assay.[1] Papain was used in the concentration of 100 mg. to 200 mg. per 2.0 gm. egg albumin. The digestion of the egg albumin was measured by the difference in volume of residue in the control tubes without papain, from those with papain.

In reviewing the work of Maher and Wirth of the National Formulary Subcommittee[17] responsible for the assay of papain, it was noted that the inadequacy of the accepted assay was acknowledged. The Subcommittee felt that any proposed assay should take into consideration (a) the uncertain identity of the active principles present; and (b) variations in raw material and in activity naturally associated with these unknown factors in commercial papain. The methods of assay studied by the Subcommittee were based upon milk coagulation, digestion of beef blood fibrin, gelatin liquefaction, and beef muscle digestion. The directness and simplicity of the principles embodied in the beef method led them to the further exploration of its possibilities for the purpose of possible improvement of the then tentatively accepted assay. The conclusions in this study were that the optimal temperature was 70° ± 2°C. and that concentrations of 0–1.5 mg. of Reference papain with 2.0 gm. dried beef powder exhibited a better relationship between concentration and activity than had been shown with 100 mg.–200 mg. papain per 2.0 gm. of beef. This work was reported in the Bulletin of the National Formulary, but apparently it did not justify making a change in the tentatively accepted assay.

The egg studies were continued, using Maher and Wirth's modification, in which the digestion temperature was 70°C. and concentration of papain was 0.1 to 0.5 mg. A remarkable amount of digestion was demonstrated with 0.1 mg. to 0.3 mg. of papain, as evidenced by the differences between the volumes of residue.

Further investigation of this proteolysis indicated the following facts: (a) a ratio of 0.1–0.3 mg. papain to 3.0 gm. of freshly coagulated egg albumin yielded a convenient, workable residue, with significant differences in volume with 0.1 mg. variations of papain; and (b) the addition of 0.25 ml. of 0.1 N sodium hydroxide per 3.0 gm. of egg albumin increased the digestion in most instances, although some egg albumin did not require the added alkali for maximum digestion. The egg albumin which did not require the added alkali for maximum digestion was not adversely influenced, however, when the alkali was added. On the basis of these findings, a new assay procedure was established which has been used successfully for more than five hundred digestions. The details of this procedure are outlined below.

Experimental. A. *Materials.* Digestion bottles—wide mouth, glass or rubber stoppered bottles of approximately 100 ml. capacity. Residue measuring vessel—100 ml. conically shaped measuring vessel which complies with the water and sediment tube ASTM Standard Method, D 96 – 35. Digestion bath—a thermostatically controlled bath capable of maintaining the temperature of the liquid at a desired point with a ±2°C. limit. Refer-

ence papain—papain reference standard as supplied by the Committee on National Formulary of the American Pharmaceutical Association. Egg albumin—the egg albumin from hen eggs which are ordinarily considered fresh. No eggs purchased, either white or brown, were found to be unsuitable.

B. *Reference Solution.* Accurately weigh sufficient papain to provide 2 mg. per cc. of standard solution. Triturate this to a smooth suspension with 5 ml. of distilled water added gradually. Transfer this suspension quantitatively to a volumetric flask, agitate thoroughly, and place in a refrigerator for 12 hours or overnight. Bring to room temperature and add water to volume. Mix and filter. Prepare a secondary dilution of the clear filtrate in distilled water of such strength that 1 ml. represents 0.1 mg. of papain. The secondary dilution should be freshly prepared for each test. The stronger solution may be stored in a refrigerator but should not be used for assay purposes after 48 hours.

C. *Preparation of Egg Albumin.* Immerse one or more fresh hen eggs in boiling water for fifteen minutes. Cool rapidly by immersion in cold water. Separate the coagulated albumin from the shell, pellicle, and yolk, and then rub it through a clean dry stainless steel No. 40 sieve, discarding the first portion. Mix the screened albumin thoroughly in a mortar and rub through the screen a second time to insure a uniform sample.

D. *Procedure.* Weigh 3.0 gm. of egg albumin into a clean dry rubber-stoppered wide-mouth bottle. Add 25 ml. of distilled water and 0.25 ml. 0.1 N sodium hydroxide solution. Mix thoroughly to break up the albumin into small particles. Take nine such bottles and add 1.0 ml., 2.0 ml., and 3.0 ml., respectively, of the standard reference solution to each of three pairs. Each pair of bottles represents 0.1 mg., 0.2 mg., and 0.3 mg. of reference papain, respectively. A solution of the papain to be assayed is prepared in a like manner and 2.0 ml. (0.2 mg. papain) are added to the fourth pair of bottles. The ninth bottle is used for a blank control with papain omitted.

Immerse bottles in a water bath at 70°C. to a point where the water in the bath is above the level of the liquid within. When the temperature has reached equilibrium, stopper the bottles. Mix at fifteen-minute intervals during digestion by slowly turning the digestion bottle one complete turn at an angle approximately 45 degrees from the vertical. At the end of two hours, transfer the undigested albumin carefully by placing a funnel in the graduated conical receiver, and pour the residue. Rinse with a jet of distilled water from a wash bottle. Bring the volume in the graduated tube to 75 ml. Allow the tubes to stand upright for two hours without agitation. Read the volume of the residues at the end of two hours to the nearest 0.1 ml. Subtract the volume of residue in each tube from the volume of residue in the control tube. The differences represent the volumes of egg albumin digested by the respective amounts of papain.

To determine the activity of the unknown papain, plot the average volume digested by 0.1 mg., 0.2 mg., and 0.3 mg. of reference papain against the logarithm of the papain concentration[23] as shown in FIGURE 1 for a representa-

tive case. The equivalent of reference papain to the volume digested is determined from the standard graph. Since 0.2 mg. of the unknown papain was used, the actual activity of the unknown in terms of reference papain is expressed by the following equation:

$$\frac{\text{equivalent concentration of unknown papain in mg.}}{0.2} \times 100 = \%\ \text{activity.}$$

Discussion

Sensitivity to Variations in Papain Concentration. In order to determine the changes in digestive activity produced by wide variations in papain concentration, experiments were set up in which the substrates (3.0 gm.

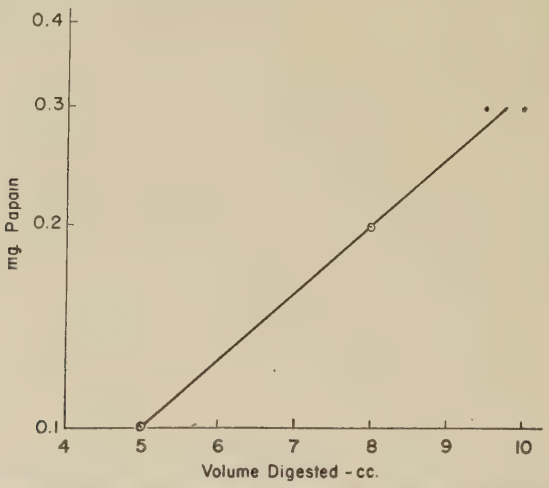

FIGURE 1. Representative standard curve for the digestion of egg albumin by National Formulary reference papain. Three grams of heat-coagulated fresh egg albumin were digested for two hours at 70°C.

egg albumin) and digestion conditions were kept constant, while the papain concentration was varied as follows: (a) from 0.1 mg. to 1.0 mg. with 0.1 mg. variations; (b) from 1.0 mg. to 10.0 mg. with 1.0 mg. variations or multiples of 1.0 mg; and (c) from 10.0 mg. to 100.0 mg. with 10 mg. variations or multiples of 10.0 mg. The assay procedure previously outlined was employed with certain modifications. The papain used was National Formulary reference papain. Several concentrations of the reference papain were prepared in order that the volume of liquid in the digestion mixture would not exceed 25 ml. in any sample. Six eggs were required to provide sufficient substrate.

The results of this study indicated an unusual phenomenon. The volume of egg albumin digested increased directly with the papain concentration from 0.1 mg. to 1.5 mg., inversely with the papain concentration from 1.5 mg. to 10 mg., and then directly with the concentration from 10 mg. to 100 mg. This is graphically illustrated in FIGURE 2, in which the logarithm of the papain concentration is plotted against the volume digested. It is

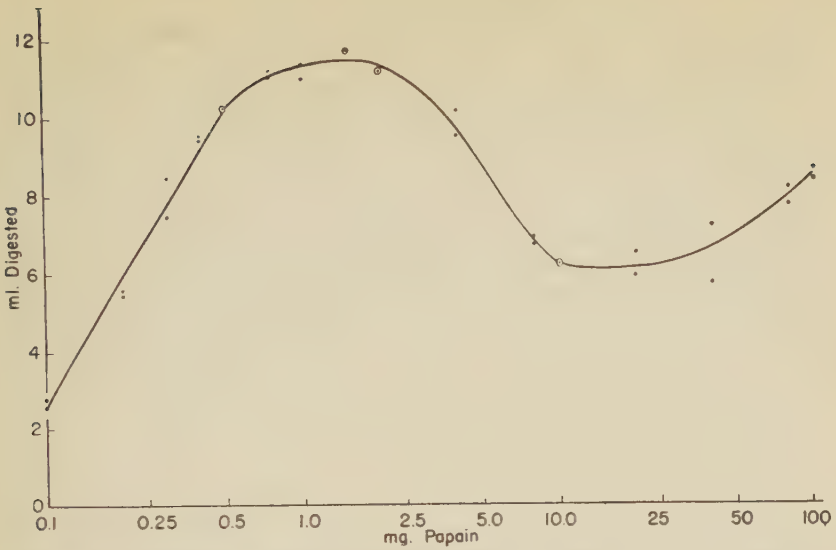

FIGURE 2. The effect of increasing the papain concentration on the digestion of 3.0 grams heat-coagulated fresh egg albumin at 70°C. for two hours.

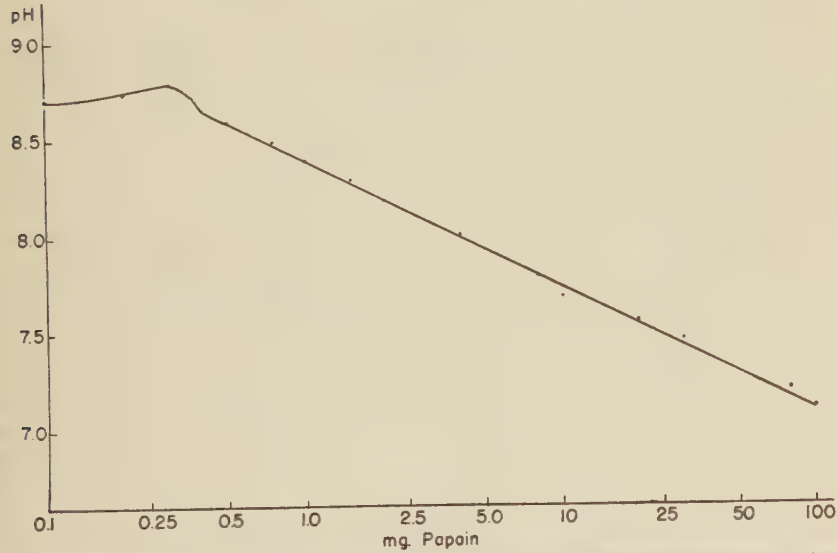

FIGURE 3. The effect of increasing the papain concentration on the pH of the digestion mixture. Digestions were carried out at 70°C. for two hours employing 3.0 grams of heat-coagulated fresh egg albumin.

apparent from this graph that the area of extreme sensitivity lies between 0.1 mg. to 0.5 mg. of papain per 3.0 gm. egg albumin.

In FIGURE 3, the pH of the digestion mixtures after digestion is plotted against the logarithm of the papain concentration. It is interesting to note that, in the area of extreme sensitivity, from 0.1 mg. to 0.4 mg., the pH

remains at 8.7 with a maximum of 8.8. As the concentration increases into the less sensitive zone, however, the pH values decrease as a straight-line function to a minimum of 7.1 at the 100 mg. concentration.

The work of Svedberg and Erikson[29] and Annets[30] on the proteolysis of egg albumin by papain has indicated that hydrolysis to amino acids is not complete, the smallest particles having an average molecular weight of several hundred. The character of the residue and of the supernatant liquid in this extended series also suggests that the hydrolysis of the albumin takes place in more than one stage. In the low concentration of papain, the residue is light and fluffy, with the supernatant liquid turbid and colloidal in appearance. In the higher concentration, however, the residue is more solid and the supernatant liquid remains almost clear.

The fact that higher concentrations of papain will digest egg, either with inverse relationship or with insufficient sensitivity, probably explains the absence of reports in recent literature on the use of egg albumin to measure papain activity.

The Effect of Activating Agents. While it is not the purpose of this paper to attempt to elucidate the activation of papain, a brief discussion is included to illustrate this phenomenon as seen with egg albumin. It has been established that there are two enzyme systems in papain.[34] Hinkel *et al.*[23, 26] and others have shown that papain possesses a native activity with such substrates as milk, casein, and gelatin, but the total potential activity of papain on these substrates is not demonstrated unless the papain is chemically activated. Gottschall[15] has reported that the presence of sulfhydryl groups can be demonstrated chemically with sodium nitroprusside in the proteolysis of beef muscle by papain. He states that the enzyme becomes progressively more active as the proteolysis proceeds, due to the liberation of previously combined sulfhydryl groups in the substrate. These facts were confirmed using dried beef powder in place of fresh beef muscle.

The application of the same test to the egg albumin substrate did not show the presence of the sulfhydryl group, however. Apparently, the activation of papain by egg albumin is due either to sulfhydryl groups insufficient for detection by this test or to the presence of some other factor or factors, insensitive to the test. The addition of hydrogen sulfide or potassium cyanide does not increase the digestion of freshly coagulated egg albumin by papain. In other words, a natural activator is present in egg albumin, or is liberated during the proteolysis, to release the total potential activity of the papain.

It has been noted in this study that papain will exert its maximum effect on egg albumin if the egg is boiled and screened immediately prior to the digestion. If the egg is allowed to stand at room temperature several hours after boiling, a marked reduction in digestion is noted. Boiled and screened egg albumin maintained for 24 hours at 4°C, 37°C., or room temperature, is not digested by 0.1–0.5 mg. papain either with or without added activators, although the same egg albumin is digested when freshly prepared. The addition of a small amount of egg yolk to the albumin does not increase the digestion in freshly prepared egg albumin, nor does it activate the papain to

cause digestion of egg albumin, which has been allowed to stand for twenty-four hours.

Other Factors Influencing Digestion. A precaution has been noted in the outline of assay that the digestion bottles be handled carefully during the digestion period and the residue in the sedimentation tubes be allowed to stand for two hours without agitation before a reading is made. This is necessary because the residue is light and fluffy, and reproducible results can be obtained only if all of the samples are handled uniformly with a minimum of agitation. It has been found that excess acid, alkali, or alkaline buffer salts in the digestion mixture will retard or inhibit digestion in the sensitive range. Maximum digestion is obtained when the pH of the digestion mixture is between 8.5 and 8.8.

The method of assay outlined is applicable to commercial papain products containing sodium bicarbonate, calcium carbonate, and alkaline bismuth or magnesium salts, if the concentration of these salts is such that the pH of the digestion mixture is not greatly changed. In the analysis of tablet mixtures, other drugs or inert excipients appeared to adsorb the enzyme to the extent that it was found preferable to use a suspension of the powdered papain-containing tablet, rather than a filtrate of the tablet mass.

Summary

(1) A method of assay for the proteolytic activity of papain is presented which is sensitive to 0.1 mg. variations in papain. According to this method, minute quantities of papain are used to digest freshly coagulated egg albumin. The amount of digestion is determined by comparing the volumes of egg albumin digested in tubes containing graded amounts of a standard preparation with those of the unknown papain.

(2) Papain is apparently activated spontaneously by egg albumin.

(3) Maximum digestion is exhibited only when the proteolysis takes place within two hours after heat coagulation of the egg albumin.

(4) An apparent reversal of digestion is observed when the ratio of papain is increased within the range of 1.5 to 10 mg. per 3.0 gm. of egg albumin.

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THE EFFECT OF THE TEMPERATURE OF DRYING PAPAYA LATEX ON THE INITIAL ACTIVITY AND STABILITY OF PAPAIN

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The fresh latex obtained from the green fruit of *Carica papaya* L. is an extremely active proteolytic agent. The relatively low activity of some commercial papain, the dried whole latex, however, indicates that a large loss of enzymes has taken place. Since papain is widely used as a source of proteolytic activity, this decrease in potency represents a large economic loss. Furthermore, papain may continue to lose activity on aging so that there may be progressive deterioration in the potency of stocks of this material.

In spite of its commercial importance, this phase of the papain problem has had little investigation. Thompson[1] studied the activity of several samples of papain varying in age from freshly prepared material to one that was six years old, and found an increasing loss of both native and total (activated) activity with age. Unfortunately, he did not give any information as to how his samples were prepared. Balls, Lineweaver, and Schwimmer[2] have reported on the initial activity and stability of vacuum dried samples of papain. They found that although some loss of both native and total activity occurs upon drying, a much greater loss of both activities takes place upon storage. Pratt[3] reported that there was very little loss of activity either by sun drying or vacuum drying fresh latex. However, he did not follow the activity of his samples on aging. Iyengar[4] reported an "11 per cent" loss in activity of a papain sample on standing for six months at room temperature. Since his method of assay, a gelatin-digestion formol titration, gives a response that is proportional to the logarithm of the enzyme concentration,[5] however, the loss of activity of his sample was actually much greater than the 11 per cent he calculated from his data. Iyengar also made no statement as to how this sample of papain was prepared.

Practically all of the papain of commerce is prepared from the fresh latex either by drying in the sun or in relatively crude hot air ovens. Sun drying is used by the natives in the jungles, although oven drying is generally employed on the estates of Ceylon and East Africa, the two major producing areas.

The latex is gathered from the green fruit in the morning and the drying process is begun in the afternoon. Sun drying requires one to several days, depending upon the weather, as opposed to oven drying, which is completed the same day. It was felt that the study of drying methods should be limited to these two, since, due to the nature of the main producing areas, vacuum drying would be almost a practical impossibility for commercial use.

The only group reporting data correlating the method of preparation and

* The assistance of Sr. J. Romagosa, of Havana, Cuba, in all phases of the collection and drying of the papaya latex used in this work, and the assistance of Mr. C. E. Alford, of these laboratories, in assaying the various samples, is gratefully acknowledged.

stability of papain[2] employed latex that had been canned and stored under refrigeration. Since no references were found to the effect of age and storage conditions upon the activity of the latex, the initial part of this study was carried out in the field. All of the latex used for this work was gathered and processed in Cuba during the late spring and early summer of 1946.

Experimental

Gathering Latex. The latex was obtained from the green papaya fruit by making several longitudinal scratches or cuts in its skin with a sharp scalpel, the free flowing latex being caught in wooden bowls. After the flow slowed down, the latex curdled along the incisions. This curdled latex was scraped from the fruit and combined with the rest of the latex. The combined latex was placed in glass containers and chilled in an ice bath at the plantation. The containers were then heavily wrapped for insulation and immediately transported to a temporary laboratory in Havana. Each lot of fresh latex was thoroughly mixed, strained through cheese cloth to remove foreign matter, and stored on ice until processed. The gathering and processing of the latex were usually done the same day.

Drying Latex. The mixed and strained latex was extruded from a glass syringe in thin spaghetti-like strings to cloth screens or trays. The extruded latex was then dried in the sun and/or in a hot air oven at 50° to 70°C. Since drying at 100°C. destroys the enzyme activity of the latex, a maximum temperature of 70°C. was used in order to minimize the effect of any local overheating in the oven. The oven-dried samples dried in about three to six hours, depending on the temperature, whereas the sun-dried samples required one to several days, depending upon the atmospheric conditions prevailing at the time.

Methods of Assay. The activity of each lot of fresh latex and each sample of papain was tested by three different methods. These methods have been discussed by us in detail elsewhere[5] and will only be mentioned here. The first was the Blau modification of the milk-clotting method, employing 25 ml. of a standard milk solution at 40°C. The results are reported as units of activity per gram (U.A./gm.) of papaya latex solids. The second was gelatin digestion measured by formol titration. One hundred milligrams of papain, or the corresponding amount of fresh latex containing 100 mg. of dry solids, was allowed to digest 20 ml. of a ten per cent gelatin solution for two hours at 40°C. The results are reported as milliequivalents of amino groups ($-NH_2$) liberated. The third was beef digestion determined by a modification[5] of Maher and Wirth's[6] method, in which 0.5 mg. of papain, or the corresponding amount of fresh latex containing 0.5 mg. of dry solids, was allowed to digest 0.5 gm. of beef powder for two hours at 70°C. The undigested residue was centrifuged and the volume of the residue was compared with that obtained from a control blank containing 0.5 gm. of beef powder but no papain. The results are reported as ml. digested per mg. of papain.

Storage. The samples of papain were placed in screw-capped glass bottles and stored at room temperature. Their activities were periodically checked by each of the three assay methods above.

Papain Solutions. For testing purposes, the papain samples were ground to pass an eighty mesh screen. A suitable quantity of the powdered sample was suspended, with the aid of glass beads in a glass stoppered bottle, in a measured amount of recently boiled, cold (less than 10°C.) distilled water. Suspensions of the latices were prepared in a similar manner. These suspensions were used within five minutes after the addition of the water to the samples in order to minimize the loss of activity that occurs in dilute papain solutions.[2, 7]

None of the samples of latex or papain were activated prior to their assay by these methods. In the absence of activators, both the milk-clotting and the gelatin-digestion methods measure only the native activity, in comparison to the beef-digestion method, which measures the total (native plus reversibly inactive) activity of a papain preparation.[8]

TABLE 1
THE INITIAL ACTIVITY OF PAPAYA LATEX AND SAMPLES OF PAPAIN PREPARED THEREFROM

Sample	*Preparation*	*Drying temperature °C.*	*Activity*		
			Milk clotting U.A./gm.	*Gelatin digestion mEq.-NH_2*	*Beef digestion ml. digested*
A	Fresh latex	—	314*	3.28*	1.50*
A-1*a*	Papain	70	213	2.29	1.26
A-1*b*	Papain	55	315	2.77	1.00
A-1*c*	Papain	Sun	322	2.82	1.30

* Based on the dry solids content.

Results

The initial activities as determined by the three methods of assay of a typical papaya latex and three samples of papain prepared from it are given in TABLE 1. The three papain samples were obtained by drying extruded whole latex in the sun and in a hot-air oven at 55° and 70°C., respectively.

The milk-clotting activity was not altered by sun drying or by oven drying at 55°C. Drying at 70°C., however, resulted in an approximate 30 per cent decrease in activity. All three drying procedures resulted in a loss of gelatin-digestion activity, and sample A-1*a* (70°C.) again showed the greatest loss. Since both the gelatin- and the beef-digestion methods give responses that are proportional to the logarithm of the enzyme concentration, and since the milk-clotting method gives a response that is directly proportional to the enzyme concentration,[5] the results obtained by the three methods cannot be directly compared. With the exception of sample A-1*a* (70°C.), these results agree fairly well with those reported by Balls *et al.*[2] and by Pratt.[3]

The effect of storage at room temperature on the milk-clotting and gelatin-digestion activities of these three papain samples is shown graphically in FIGURES 1-A and 1-B, respectively. It can be seen that both the milk-clotting and the gelatin-digestion activities fall with age. Sample A-1*a*, how-

ever, which was dried at 70°C. and which had the lowest initial activity, has a significantly greater activity after approximately two years of storage than the other two. The higher drying temperature seems to enhance the stability.

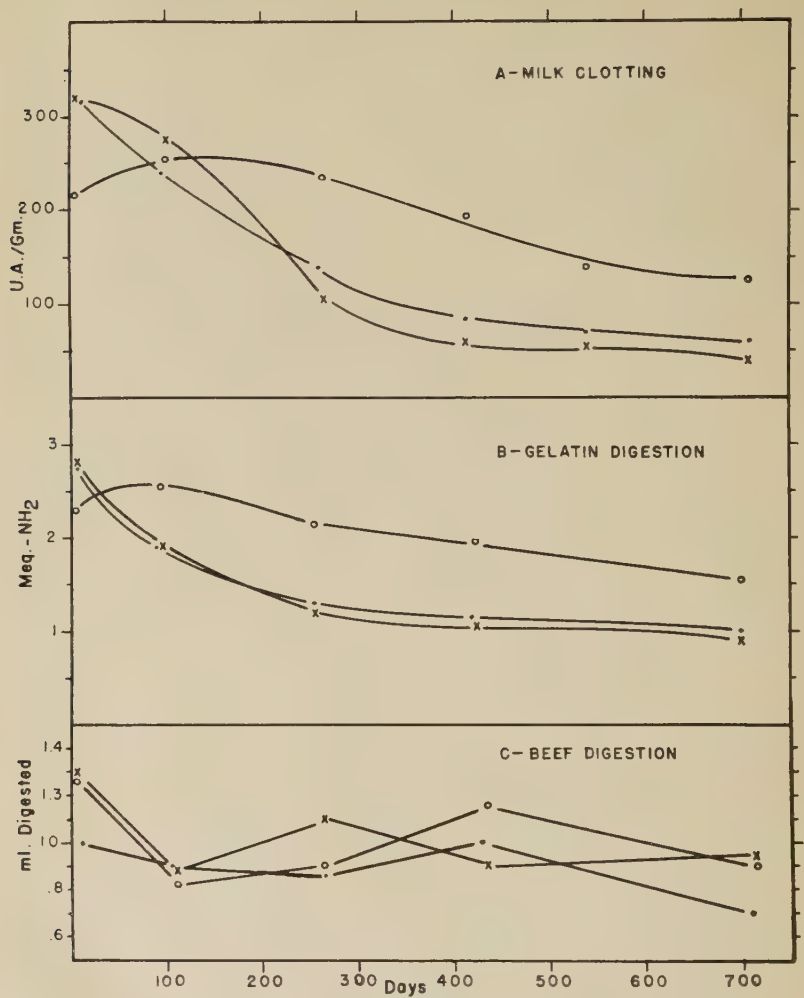

FIGURE 1. The effect of storage at room temperature on the activity of papain prepared from fresh latex by drying at 70°C. (Sample A-1*a*, ○), at 55°C. (Sample A-1*b*, ●), and in the sun (Sample A-1*c* ×).

The effect of aging on the beef-digestion activity is shown in FIGURE 1-C. The results are more irregular but they show an overall decrease in activity for all three samples. The decrease is much less than that found by the milk-clotting and gelatin-digestion methods, however. These results agree fairly well with those of Thompson,[1] in that the native activity falls off faster than the total activity.

As mentioned earlier, no references were found to the effect of storage on the activity of fresh latex. Since quick freezing has been used so extensively and with good results in the preservation of fresh foodstuffs, it was felt that it offered a possible means by which fresh papaya latex might be stored and transported. Consequently, a quantity of fresh latex was divided into three portions, two of which were quick-frozen and stored in a Havana frozen food processing plant. The third portion was assayed by the three methods in order to obtain the initial activity of the latex as a control. The activity of one of the portions of frozen latex was determined after two weeks of frozen storage and the other after one month of frozen storage. The excess latex in each case was oven dried at 55–60°C.

The initial activities of these three latices and of the two samples of papain derived from them after drying are given in TABLE 2. It can be seen that quick freezing and subsequent frozen storage for at least a month has no

TABLE 2

THE EFFECT OF QUICK FREEZING AND STORAGE IN THE FROZEN STATE ON THE ACTIVITY OF PAPAYA LATEX AND THE INITIAL ACTIVITY OF PAPAIN PREPARED THEREFROM

Sample	*Preparation*	*Activity*		
		Milk clotting U.A./gm.	*Gelatin digestion mEq.-NH_2*	*Beef digestion ml. digested*
B	Fresh latex	344*	3.48*	1.12*
B-1	Latex frozen 2 weeks	392*	3.52*	1.15*
B-2	Latex frozen 1 month	299*	3.41*	1.25*
B-1*a*	Latex B-1, dried 55–60°C.	297	2.92	0.86
B-2*a*	Latex B-2, dried 55–60°C.	397	3.00	1.10

* Based on the dry solids content.

effect on the activity of papaya latex. There was a slight drop in activity upon drying, but no greater than that found on drying fresh unfrozen latex.

The loss of activity upon aging papain samples B-1*a* and B-1*b*, prepared from two-weeks- and one-month-old frozen latex respectively, are shown in FIGURE 2. There is a rather rapid initial fall followed by a slow decline in native activity (FIGURES 2A and 2B). The total activity, however, as measured by the beef method, appears to be fairly stable. These results are similar to those obtained with unfrozen latex and again indicate that freezing does not have any deleterious effect on the proteolytic enzymes of papaya latex. In subsequent experiments, latex has been kept in the frozen state for over three years without loss of activity, and the latex, after thawing, still had a fresh odor and appearance.

Balls and coworkers[9] have reported that press juice, the juice from the pulp of the papaya fruit, has an inactivating effect on the proteolytic enzymes of the latex. It seemed possible that if the fruit were cut too deeply when gathering the latex the latter might be contaminated with juice from the pulp, and its enzymes thereby partially destroyed. Consequently,

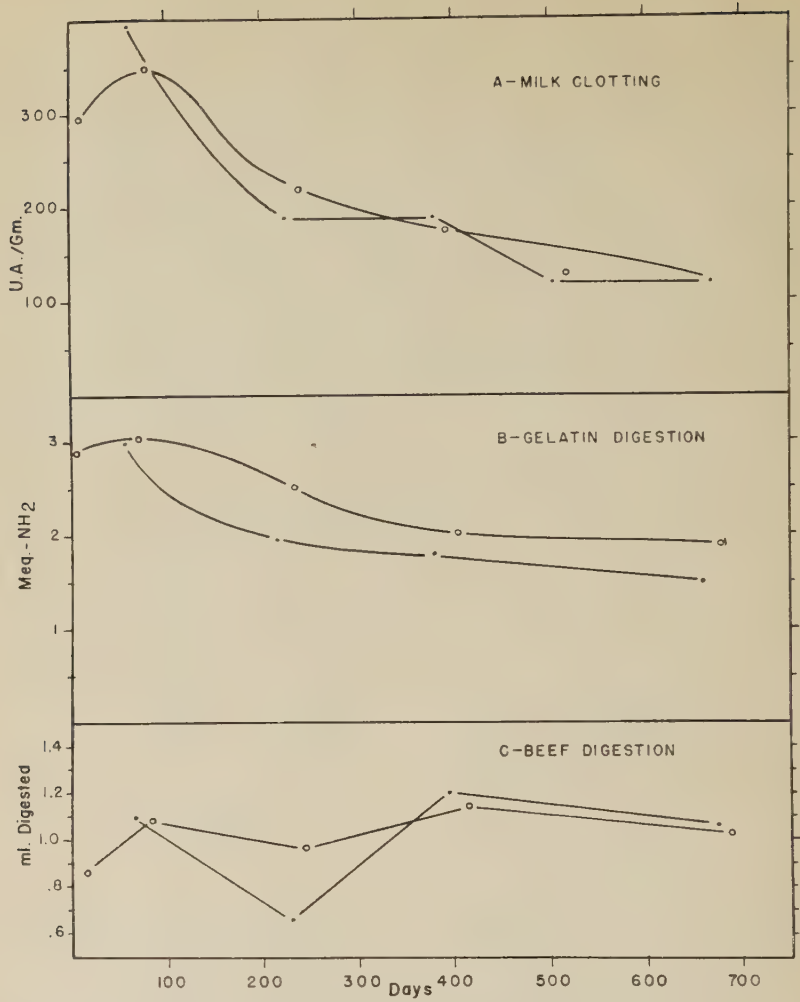

FIGURE 2. The effect of storage at room temperature on the activity of papain prepared from latex held in the frozen state for two weeks (Sample B-1*a*, ○) and one month (Sample B-2*a*, ●).

TABLE 3

THE INITIAL ACTIVITY OF PAPAYA LATEX OBTAINED FROM DEEP-CUT FRUIT AND PAPAIN PREPARED THEREFROM

Sample	*Preparation*	*Activity*		
		Milk clotting U.A./gm.	*Gelatin digestion mEq.-NH_2*	*Beef digestion ml. digested*
C	Latex	279*	3.25*	1.02*
C-1*a*	Latex, dried 55°C.	231	3.00	1.02
C-1*b*	Latex, sun-dried	163	2.71	0.80

* Based on the dry solids content.

latex gathered by making deep incisions in the green fruit was both sun dried and oven dried at 55°C. The initial activities of this latex and the two papain samples prepared from it are given in TABLE 3. By comparing

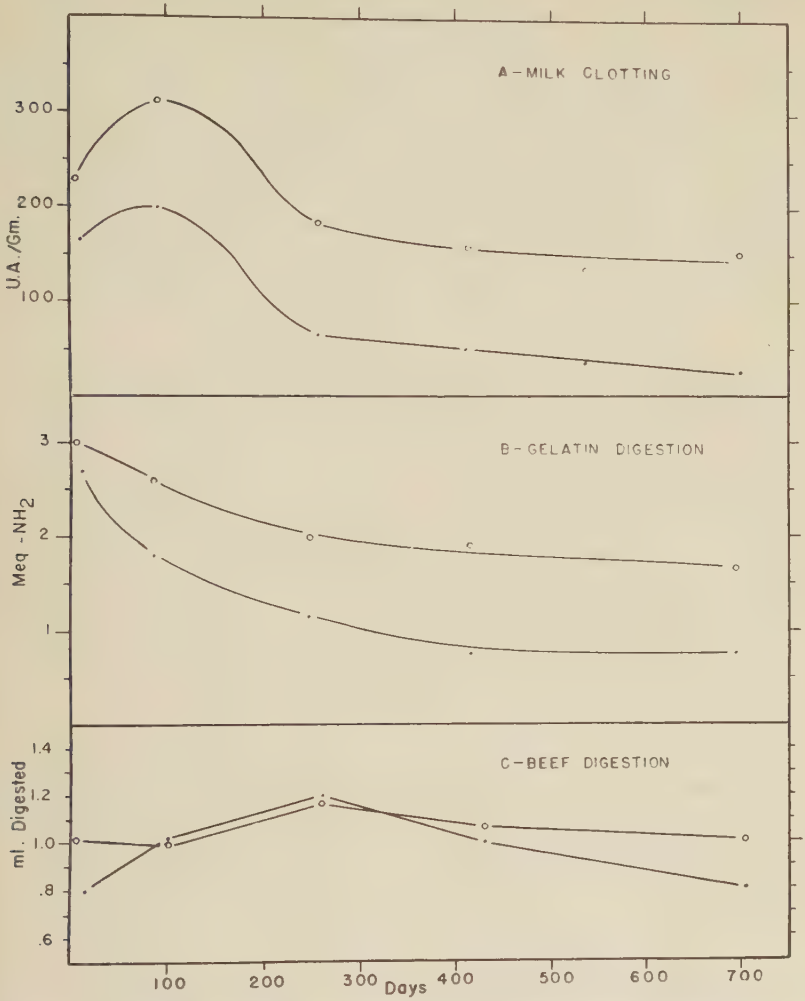

FIGURE 3. The effect of storage at room temperature on the activity of papain prepared from latex obtained from deeply cut fruit by drying at 50°C. (Sample C-1*a*, ○) and in the sun (Sample C-1*b*, ●).

these results with those given in TABLES 1 and 2, it can be seen that the depth of the cut used to tap the fruit did not affect the activity of the latex obtained. The results of sun drying, however, do not agree with those for latex series A. Sample C-1*b* shows a definite loss of activity on sun drying.

The relative stabilities of these two papain samples during storage are shown in FIGURE 3. The activities of the sun-dried samples C-1*b* are significantly less than those of the oven-dried sample. This is especially

noticeable for the milk-clotting and gelatin-digestion activities, FIGURES 3A and 3B. As a matter of fact, the native activity of the sun-dried sample had practically disappeared after approximately two years of storage at room temperature.

All of the above experimental papains were very light in color, the darkest having only a slight cream cast. Commercial papain, on the other hand, ranges in color from an off-white to a dark brown, and in general its activity varies inversely with the color. Examination of several samples of unground papain showed that in some commercial material the latex had been dried in rather thick layers, and that the darker samples showed evidence of having been dried in the thickest layers. Since the present experimental samples had been dried in very thin layers, it was thought that their differences from commercial material in color and activity might be due to the slower drying which occurred when the latex was spread in thick layers.

TABLE 4

THE EFFECT OF DRYING PAPAYA LATEX IN VARYING THICKNESSES ON THE INITIAL ACTIVITY OF PAPAIN

Sample	*Preparation*	*Activity*		
		Milk clotting U.A./gm.	*Gelatin digestion mEq.-NH_2*	*Beef digestion ml. digested*
D	Fresh latex	251*	3.71*	1.10*
D-1*a*	Thin layers, dried 50°C.	209	2.70	0.92
D-1*b*	Thick layers, dried 50°C.	233	2.66	0.88
D-2*a*	Thin layers, sun-dried	179	2.31	1.02
D-2*b*	Thick layers, sun-dried	125	2.11	0.92

* Based on the dry solids content.

An experiment was, therefore, carried out on the effect of drying papaya latex in varying thicknesses. A sample of fresh latex was both oven-dried and sun-dried in thick (about $\frac{1}{2}$ inch) and thin (about $\frac{1}{8}$ inch) layers. The thick samples required much longer to dry. The oven-dried samples exhibited no difference in color, and the thick sun-dried sample (D-2*b*), although slightly darker than the others, was not so dark as some of the darker commercial samples. Sample D-2*b* did, however, develop a very offensive odor, indicating that some decomposition had taken place. The initial activities of these samples, as well as the initial activities of the fresh latex from which they were prepared, are given in TABLE 4. Sun drying again adversely affected the milk-clotting and gelatin-digestion activities. The thickness of the latex did not seem to have had any effect except in the case of D-2*b* (sun-dried), which had shown signs of decomposition. Once again it can be seen that the beef-digestion activity was not appreciably altered by the drying method. This would suggest that the major change occurring in the activity under these varying conditions was a shift from the "native active" to the "reversibly inactive" form of the enzymes.

The effect of storage on the activities of these samples is shown in FIGURE

4. Here the detrimental effect of sun drying on the native activity of papain is again seen. It is interesting to note that although sample D-2*b* (sun-dried, thick) required a much longer time to dry than D-2*a*, its activity (FIGURES 4A and 4B) did not fall to quite so low a level. This might be the

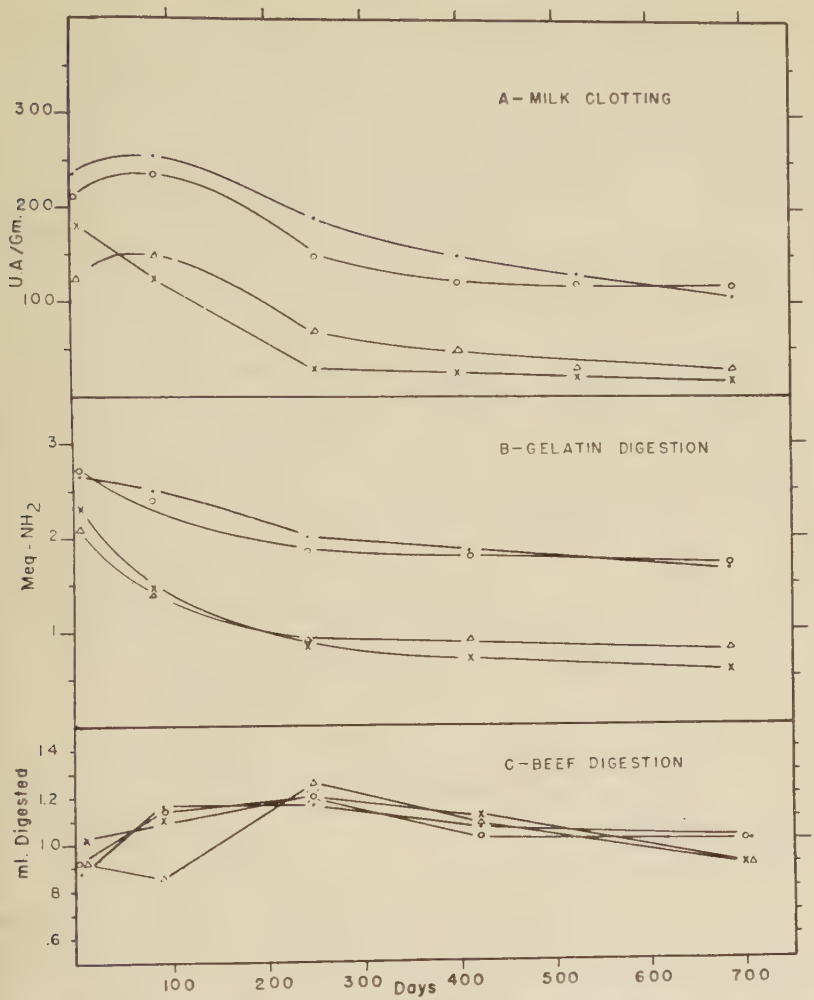

FIGURE 4. The effect of storage at room temperature on the activity of papain prepared from fresh latex by drying in thick layers at 50°C. (Sample D- *b*, ●) and in the sun (Sample D-2*b*, △); and in thin layers at 50°C. (Sample D-1*a*, ○) and in the sun (Sample D-2*a*, ×).

result of the greater thickness of sample protecting some of the material from the sun. The beef-digestion activities, FIGURE 4C, again show that the total activity is relatively stable.

Summary

Oven drying of papaya latex results in little loss of initial proteolytic activity, as has also been shown previously by Balls *et al.*[2] and by Pratt.[3]

Contrary to the latter report, however, it was found in the present studies that sun drying results in a loss of native activity. All samples of papain showed large decreases in native activity on storage at room temperature for periods of approximately two years, the sun-dried samples being the least stable.

The total activity, as measured by beef digestion, was surprisingly stable. This was not anticipated from the results of previous investigators.[1, 2] The high beef digestion values result in part from measuring the action of the reversibly inactive state of the enzymes.

The ability to store fresh papaya latex in the frozen state for periods of three years or more without any noticeable change in either appearance or activity should prove useful in future studies of this material.

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THE EFFECT OF CHEMICAL TREATMENT OF PAPAYA LATEX ON THE INITIAL ACTIVITY AND STABILITY OF PAPAIN

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The effect of variations in the method of drying papaya latex, on the initial activity and stability of papain, has been recently reported from these laboratories.[1] Although several other investigators[2–5] have also discussed this phase of the papain problem, only two[2, 4] have published the results of any experiments on treatment of papaya latex prior to drying. Pratt[2] prepared a concentrate from fresh latex by precipitating it with alcohol, washing with ether, and then drying. This product had a higher activity than fresh latex. Unfortunately, nothing was reported concerning its stability. Balls and coworkers[4] treated papaya latex with sodium sulfide, sodium cyanide, and sodium chloride. They found that, when the treated latices were vacuum-dried, the resulting products were little if any more active or more stable than air-dried, untreated latex. On the other hand, when the sodium chloride-latex was only partially dried, the resulting paste was quite active and showed only about 10 per cent loss in activity when kept in sealed containers for 190 days. Contrary to this, however, when both sodium chloride and sodium sulfide were added to latex and this mixture was partially dried, the resulting paste showed none of these favorable storage characteristics. Because of the paucity of data on the effects of chemical treatment of the latex on potency and stability of the enzyme, it was thought desirable to extend the observations in this field to determine if papains of improved characteristics could be produced.

Experimental

Latex was gathered from green papaya fruit and dried, unless stated otherwise, in the same manner as described previously.[1] Prior to drying, however, the latex was thoroughly mixed with the desired reagent.

The proteolytic activity was measured by the same three methods (milk clotting, gelatin digestion formol titration, and beef digestion) employed before[1] and discussed critically elsewhere.[6, 7] The Blau modification of the milk-clotting method, employing 25 ml. of a standard milk solution at 40°C., was used. The results are reported as units of activity per gram (U.A./gm.) of papaya latex solids. Gelatin digestion was followed by formol titration. One hundred milligrams of papain or the corresponding amount of fresh latex containing 100 mg. of dry solids was allowed to digest 20 ml. of 10 per cent gelatin solution for two hours at 40°C. The results are reported as milliequivalents of amino groups liberated. Beef digestion was determined by allowing 0.5 mg. of papain or the corresponding amount of fresh latex to digest 0.5 gm. of beef powder for two hours at 70°C. The undigested residue was centrifuged and the volume of residue compared

* The assistance of Sr. J. Romagosa, of Havana, Cuba, in the collection and drying of the latex used in this work, and the assistance of Mr. C. E. Alford, of these laboratories, in assaying the samples, is hereby gratefully acknowledged.

with that obtained from a control blank containing 0.5 gm. of beef powder but no enzyme. The results are reported as ml. of beef digested per mg. of papain. None of the samples were activated before testing. Consequently, both the milk-clotting and gelatin-digestion methods measure the native activity, while the beef digestion measures the total (native plus reversibly inactive) activity.[7] The samples of papain were stored at room temperature in screw-capped glass bottles. Their activities were periodically checked by each of the three assay methods for approximately two years.

Since the inactivation of papain on aging is generally believed to be connected with an oxidation process, the possible protective action of two re-

TABLE 1
THE EFFECT OF THE CHEMICAL TREATMENT OF PAPAYA LATEX ON THE INITIAL ACTIVITY OF DRIED LATEX

Sample	*Preparation*	*Activity**		
		Milk clotting U.A./gm.	*Gelatin digestion mEq.-NH_2*	*Beef digestion ml. digested*
A	Fresh latex	314	3.28	1.50
A-2*a*	Latex + 2% $Na_2S_2O_3$ dried 70°C.	70	1.69	1.33
A-2*b*	Latex + 2% $Na_2S_2O_3$ dried 55°C.	169	2.43	0.95
A-3*a*	Latex + 2% $NaHSO_3$ dried 70°C.	92	1.52	1.07
A-3*b*	Latex + 2% $NaHSO_3$ dried 55°C.	322	2.71	0.80
A-4*a*	Latex + 2% citric acid dried 70°C.	149	1.66	1.04
A-4*b*	Latex + 2% citric acid dried 55°C.	258	2.50	0.84

* All activities are calculated on the basis of dry papaya latex solids.

ducing agents, sodium thiosulfate and sodium bisulfite, was investigated. Two per cent by weight of the respective chemicals was added to portions of a fresh latex and stirred until dissolved. The resulting mixtures were then subdivided and dried in a hot air oven at 55° and 70°C. Samples were also placed out to sun-dry, but these were destroyed by a sudden tropical storm. Another set of samples was prepared at the same time from latex treated, in a like manner, with two per cent by weight of citric acid, since Balls *et al.*[8] had found that the acidification of papaya leaf juice aided in the inhibition of the oxidizing processes resulting in the inactivation of the enzyme. These chemicals in this proportion liquefied the latex so that it had to be dried in shallow glass trays.

The initial activities of these preparations are given in TABLE 1. It can be seen that sample A-3*b* (two per cent bisulfite, 55°) had by far the highest milk clotting activity and was also the most active by the gelatin-digestion method. The beef-digestion results do not agree with those obtained by the other methods. It should be pointed out, however, that the beef-

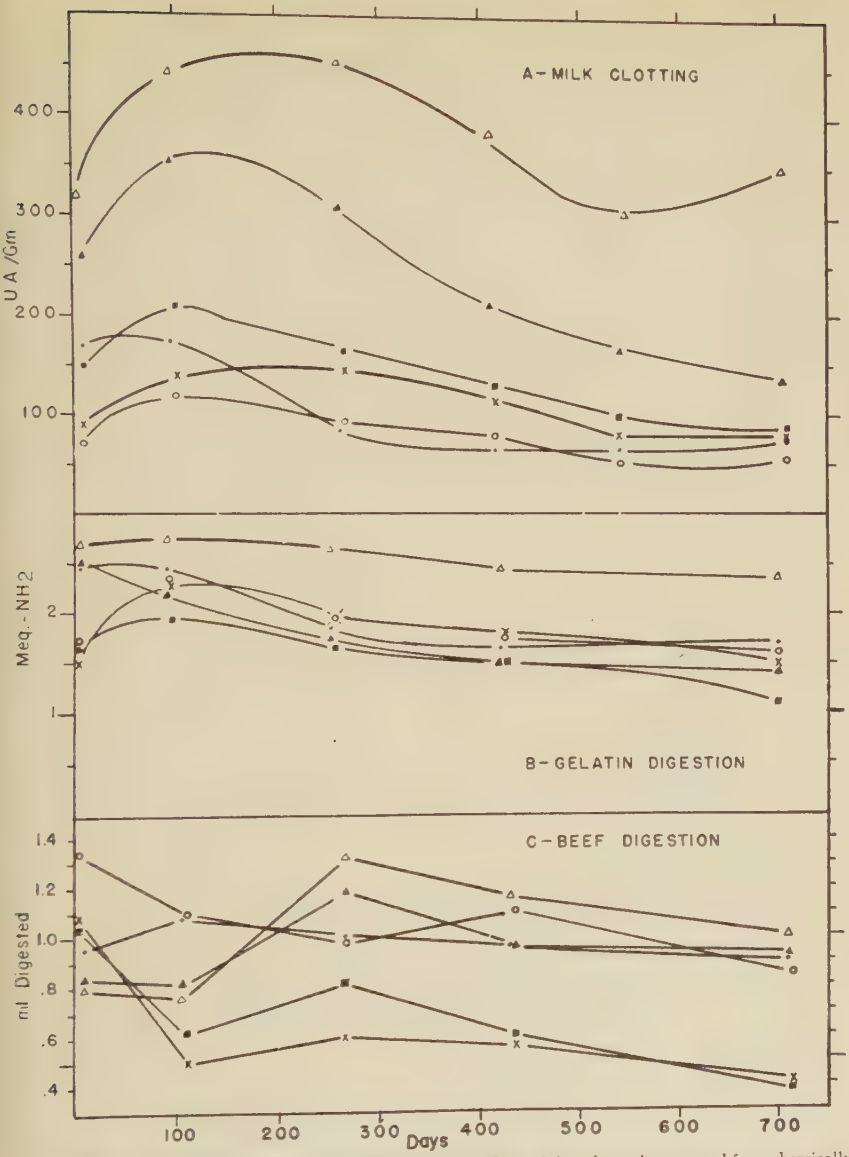

FIGURE 1. The effect of storage at room temperature on the activity of papain prepared from chemically treated fresh latex. Latex plus two per cent sodium thiosulfate, dried at 70°C. (Sample A-2*a*, ○) and at 55°C. (Sample A-2*b*, ●); two per cent sodium bisulfite dried at 70°C. (Sample A-3*a*, ×) and at 55°C. (Sample A-3*b*, △); two per cent citric acid, dried at 70°C. (Sample A-4*a*, ■) and at 55°C. (Sample A-4*b*, ▲).

digestion activity seems to be adversely affected by drying at the lower temperature, while both the milk-clotting and the gelatin-digestion activities are adversely affected by the higher temperature. We are unable to give any explanation for this difference.

The effect of storage on the milk-clotting activity of this series of samples is shown in FIGURE 1A. The stability of A-3*b* (two per cent bisulfite, 55°) is quite striking, its activity after approximately two years of storage being slightly greater than that of the fresh latex from which it was prepared. The next best sample, A-4*b*, lost approximately 50 per cent of its activity in this same length of time. The stability of the remainder of this series is disappointing, being even less than papain prepared from the untreated latex by drying at 70°C. (reference 1, FIGURE 1). The increased milk-clotting activity after aging for 100 days is puzzling and cannot be accounted for.

The surprising stability of sample A-3*b* is also evident in FIGURE 1B, which shows the effect of aging on the gelatin digestion activity of this series, and is evident again in FIGURE 1C, which gives the data for the beef-digestion

TABLE 2

THE INITIAL ACTIVITY OF CRUDE ALCOHOL PRECIPITATED CONCENTRATES AND OF A DRIED PRESS JUICE—LATEX MIXTURE

Sample	*Preparation*	*Initial activity*		
		Milk clotting U.A./gm.	*Gelatin digestion mEq.-NH_2*	*Beef digestion ml. digested*
B	Fresh latex	344*	3.48*	1.12*
B-3	Concentrate, alcohol precipitate of B.	385	3.21	1.06
D	Fresh latex	251*	3.71*	1.10*
D-5	Concentrate, alcohol precipitate of D.	240	2.60	1.16
C	Fresh latex	297*	3.25*	1.02*
C-2	Latex C plus 5% press juice	262	2.89	0.90

* Based on the dry solids content.

activities. Except for the irregular beef-digestion activity exhibited at the start, this sample was consistently more active than any of the others.

Balls and Lineweaver[9] have reported on the relatively high stability of crystalline papain. It would seem that material partially purified by alcohol precipitation might show an increased stability of activity. Two samples, therefore, were prepared by the addition of four volumes of 95 per cent alcohol to one volume of fresh latex. The supernatant liquid was decanted and the precipitate washed with alcohol and then dried. The initial activities of these two samples and the fresh latices from which they were prepared are given in TABLE 2. Contrary to a previous report,[2] the activity was not increased by this treatment. The effect of aging on the activities of these samples is shown in FIGURE 2. Except for the accelerated loss of milk-clotting and gelatin-digestion activity exhibited by sample B-3, the results are similar to those obtained for papain prepared from untreated latex.[1]

TABLE 2 also shows the initial activity of a sample of papain prepared from a mixture of fresh latex and the juice of the pulp of the fruit. A green

papaya fruit was tapped and the latex allowed to drain from it. Pulp from this bled fruit was grated to facilitate the collection of the juice. This pulp juice or press juice was then mixed with fresh latex in the proportion of 1:20.

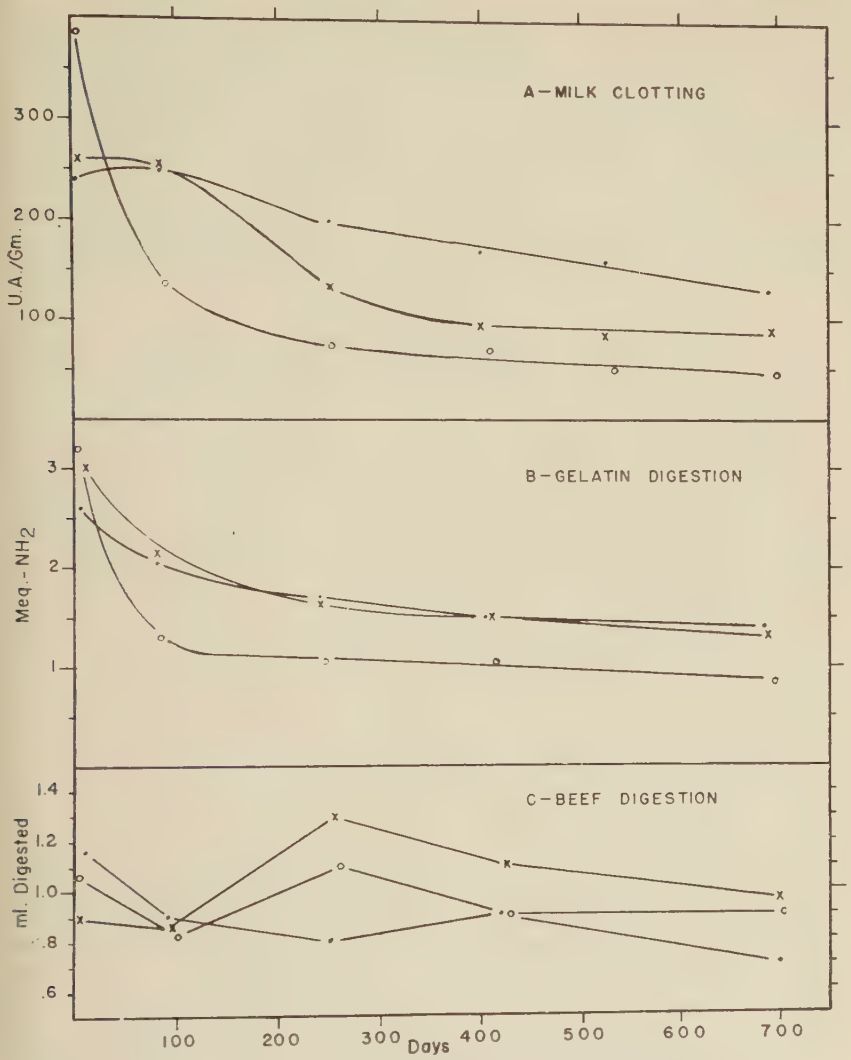

FIGURE 2. The effect of storage at room temperature on the activity of alcohol precipitated concentrates (Samples B-3, ○, and Sample D-5, ●) and of dried press juice-latex mixture (Sample C-2, ×).

This mixture was allowed to stand at room temperature for 30 minutes before drying at 50°C. There was no inactivation of the enzyme by this treatment. Balls *et al.*,[8] on the other hand, reported that press juice rapidly inactivated latex when the two were mixed. The effect of aging on the activity of this preparation is also shown in FIGURE 2. The results are similar to those obtained for papain prepared from untreated latex.[1]

Previously,[1] experiments were described concerning the effect of drying latex in layers of varying thicknesses. As an extension of that work, latex treated with 0.5 per cent of sodium bisulfite was both oven- and sun-dried in thin ($\frac{1}{8}$ inch) and thick ($\frac{1}{2}$ inch) layers. The initial activities of these samples of papain are given in TABLE 3. In contrast to the results obtained with untreated latex, there was little if any loss of activity on drying. The thick sun-dried sample (D-4*b*) did not show any of the evidences of decomposition that were so apparent with the corresponding sample prepared from untreated latex.[1] The bisulfite no doubt acted as a preservative and protected the latex from bacterial decomposition.

The stability of these samples is shown in FIGURE 3. Two things are readily apparent: sample D-3*a* (thin, 50°) shows the same high degree of

TABLE 3

THE EFFECT OF THE THICKNESS OF LATEX AND MANNER OF DRYING ON THE INITIAL ACTIVITY OF PAPAIN PREPARED FROM LATEX TREATED WITH 0.5 PER CENT SODIUM BISULFITE

Sample	*Preparation*	*Activity**		
		Milk clotting U.A./gm.	*Gelatin digestion mEq.-NH_2*	*Beef digestion ml. digested*
D	Fresh untreated latex	251	3.71	1.10
D-3*a*	Treated latex, dried 50°C., thin layer	272	2.83	1.06
D-3*b*	Treated latex, dried 50°C., thick layer	262	2.61	1.02
D-4*a*	Treated latex, sun-dried, thin layer	292	2.82	1.02
D-4*b*	Treated latex, sun-dried thick layer	283	3.00	1.12

* All activities are calculated on the basis of dry papaya latex solids.

stability exhibited by sample A-3*b* above, and the detrimental effect of sun drying noted before[1] is again quite evident. The extended length of time that sample D-3*b* was exposed in the semi-liquid state to an elevated temperature, due to its slower drying, might have caused the decomposition of the bisulfite, and thereby may explain why this sample exhibited a typical untreated papain decrease in native activity on aging.

There is a decided initial hump in the milk-clotting activity curves, FIGURE 3A, the magnitude of which corresponds to the initial activity of the preparation. This same type of results was also noted previously.[1] As far as is known, this increase in activity with moderate aging has not been reported by other investigators. No explanation for this unexpected behavior can be offered at this time.

Summary

The effect of the chemical treatment of papaya latex on the initial activity and stability of papain has been investigated. Sodium bisulfite has a

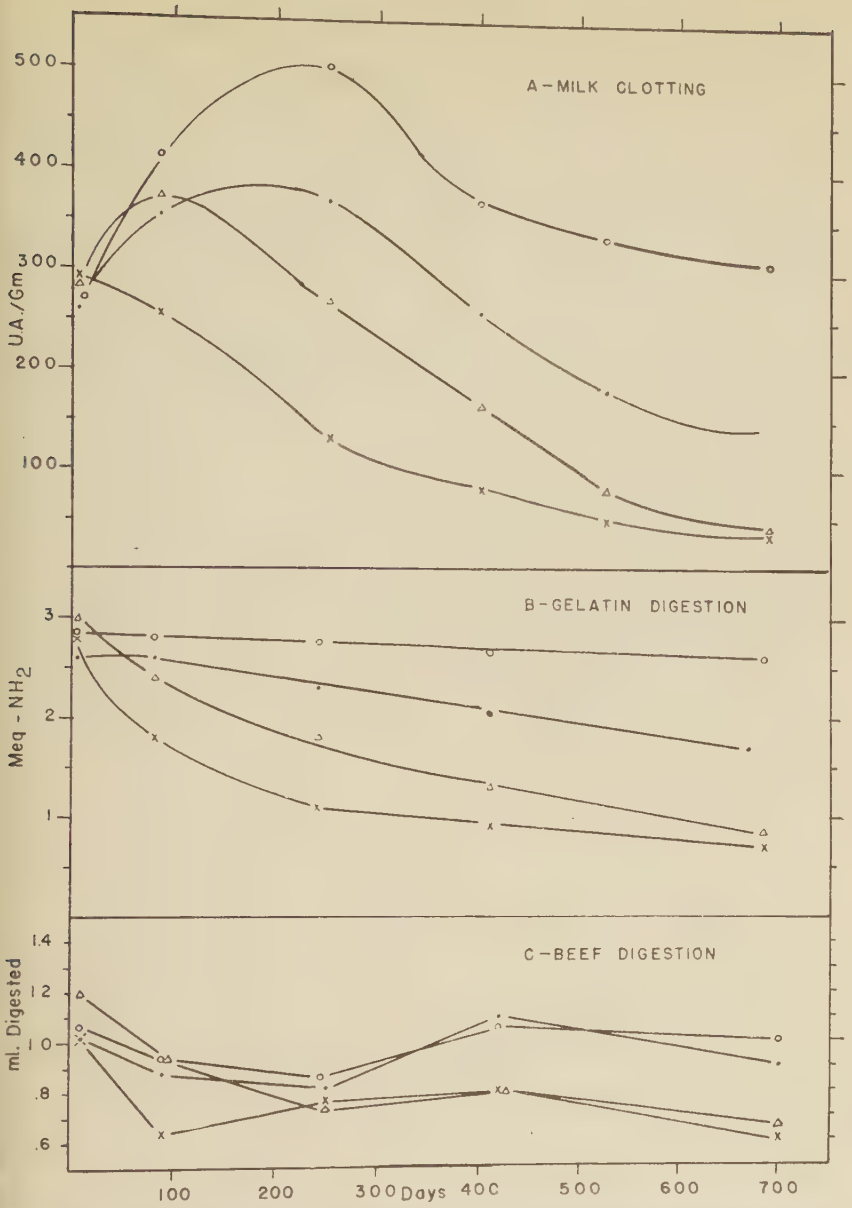

FIGURE 3. The effect of storage at room temperature on the activity of papain prepared from fresh latex treated with 0.5 per cent sodium bisulfite by drying in thick layers at 50°C. (Sample D-3*b*, ●) and in the sun (Sample D-4*b*, △); and in thin layers at 50°C. (Sample D-3*a*, ○) and in the sun (Sample D-4*a*, ×).

marked stabilizing effect on the proteolytic activity of papain. Two samples of bisulfite-treated papain showed no loss in activity by milk clotting or gelatin or beef digestion after approximately two years of storage at room

temperature. This stabilizing influence, however, is more than offset by the detrimental effect of the sun on sun-dried papain.

Samples of papain prepared from latex containing either sodium thiosulfate or citric acid were not more stable than dried untreated latex. Partial purification by means of alcohol precipitation of fresh latex also resulted in products without improved stability.

The addition of five per cent of the juice from the pulp of the green papaya fruit to fresh latex did not produce any noticeable inactivation of the enzyme in the resulting dry product.

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FURTHER STUDIES ON THE EFFECT OF DRYING CONDITIONS AND OF THE CHEMICAL TREATMENT OF PAPAYA LATEX ON THE STABILITY OF PAPAIN

By E. T. Hinkel, Jr.*
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A recent communication from these laboratories[1] has shown the effect of various methods of drying papaya latex on the initial activity and stability of papain. A subsequent report[2] has also been made of the effects of chemical treatment of latex prior to drying. It was found that addition of sodium bisulfite to the latex produced, in some cases, a beneficial effect on both the initial activity and stability of the resulting papain. The present paper reports the continuation of these studies on the drying conditions and the chemical treatment of fresh latex.

Methods

The latex was gathered and dried in Cuba by methods that have already been described.[1] Chemical treatment consisted of adding the desired amount of the compound to the fresh latex and stirring until it had either been completely dissolved or evenly dispersed therein.

The proteolytic activity was measured by the same three methods employed before,[1, 2] namely the Blau modification[3] of the Balls and Hoover[4] milk-clotting method, gelatin digestion-formol titration, and beef digestion. None of the samples were activated prior to testing. These methods have been critically compared elsewhere,[3, 5] and it will suffice to say here that both the milk-clotting and the gelatin-digestion methods measure only the native or naturally active enzyme, while the beef digestion method measures the total, or native, plus the reversibly inactive enzyme under these conditions. All three measure true proteolysis.[5]

Experimental

Twenty-eight hundred grams of thoroughly mixed and strained freshly gathered papaya latex were divided into seven equal parts. The first part was left untreated as a control, the second part was mixed with 0.2 per cent of powdered thymol, the third part with 0.1 per cent of powdered thymol, the fourth part with 0.2 per cent of powdered thymol plus 0.5 per cent of sodium bisulfite, the fifth portion with 0.1 per cent each of thymol and sodium bisulfite, the sixth part with 0.1 per cent of sodium bisulfite, and the last part with 10 per cent of sodium chloride. This last treatment was included because of the favorable results reported by Balls and coworkers[6] when mixtures of salt and latex were partially dried to a paste.

Each lot of latex was then subdivided into six, approximately 65-gram, portions for drying. One portion of each of the seven lots of latex was dried according to the following schedule: (*a*) dried immediately at 70°C;

* The assistance of Sr. J. Romagosa, of Havana, Cuba, in the collection and drying of the latex used in this investigation, and of Mr. C. E. Alford, of these laboratories, in assaying the samples, is hereby gratefully acknowledged.

(*b*) stood three hours at room temperature and then dried at 50°C; (*c*) sun dried immediately; (*d*) stood 24 hours at room temperature and then dried at 70°C; (*e*) stood 27 hours at room temperature and then dried at 50°C; and (*f*) stood 24 hours at room temperature and then sun dried.

The time lag between the 70° and 50° drying schedules was necessitated by the fact that there was only one reliable oven of suitable capacity available. It took three hours to dry the 70° sample and readjust the oven to 50°. A sudden tropical rain unfortunately destroyed the *c* set of samples completely.

Initial Activity. The initial activities of the papain samples and of the fresh latex itself are given in TABLE 1. There was much less inactivation of the untreated samples resulting from the room temperature holding period than was expected. The adverse effect of sun drying is clearly seen in the milk-clotting and gelatin-digestion values of E-1*f*.

The treatment with 0.2 per cent of thymol, series E-2, although having no effect on the loss of activity accompanying the drying of the fresh latex, counteracted the adverse effect of the 24-hour holding period noted for the untreated series, E-1. As usual, the sun-dried sample had the lowest activity.

Decreasing the amount of thymol used to treat the fresh latex from 0.2 per cent to 0.1 per cent, series E-3, had no noticeable effect. The initial activities of this series are practically identical with those for the E-2 series.

The addition of 0.5 per cent of sodium bisulfite and 0.2 per cent of thymol, series E-4, however, produced a different result. The milk-clotting activity of all five samples was higher than that of the fresh latex, and the gelatin and beef digestion activities showed less loss than for the preceding series. Even the adverse effect of sun drying was counteracted.

Reducing the thymol and bisulfite content to 0.1 per cent of each, series E-5, had a detrimental effect upon the native activity of the sun-dried sample, E-5*f*. The other samples compare favorably with those of series E-4.

Sodium bisulfite, 0.1 per cent, series E-6, was not as effective in protecting the initial milk-clotting activity as when used with thymol. Otherwise, the activities for the oven-dried samples were very similar to those obtained in the E-4 and E-5 series.

The addition of 10 per cent sodium chloride to the fresh latex prior to drying, series E-7, produced essentially the same effect on the initial activity of the resulting papain as was obtained with the thymol treatment, series E-2 and E-3.

Stability. The effect of storage at room temperature on the activities of the E-1 series of samples, prepared from untreated latex, is shown in FIGURE 1. The milk-clotting activity, FIGURE 1A, shows the same pattern as has been reported before,[1, 2] an unexplained rise followed by a steady fall. It will be noted that the two samples that were oven-dried after standing for 24 hours (E-1*d* and E-1*e*) had consistently lower activities than the two dried the first day. As usual, the sun-dried sample, E-1*f*, having the lowest initial activity, also had the least activity at the end of approximately two years. The gelatin-digestion activities, FIGURE 1B, closely follow the milk-

TABLE 1

THE INITIAL AND 700-DAY ACTIVITY OF PAPAIN PREPARED FROM BOTH TREATED AND UNTREATED PAPAYA LATEX

Series and treatment prior to drying	*Sample and drying conditions*	*Activity* (as per cent of fresh latex)*					
		Milk clotting		*Gelatin digestion*		*Beef digestion*	
		initial	*700 days*	*initial*	*700 days*	*initial*	*700 days*
E	fresh latex	100		100		100	
E-1, fresh latex untreated	*a*, dried immed. 70°	85.6	43.0	83.3	59.5	89.9	97.0
	b, dried after 3 hrs., 50°	91.5	43.6	80.5	53.7	76.8	94.9
	d, dried after 24 hrs., 70°	70.8	27.5	76.4	43.1	66.7	90.9
	e, dried after 27 hrs., 50°	71.5	31.1	77.9	46.3	90.9	90.9
	f, dried after 24 hrs., sun	54.1	16.1	68.7	26.7	66.7	64.6
E-2, fresh latex plus 0.2% thymol	*a*, dried immed. 70°	86.9	34.1	81.3	44.3	102.0	90.9
	b, dried after 3 hrs., 50°	88.5	29.2	77.9	39.1	70.7	101.0
	d, dried after 24 hrs., 70°	83.6	43.6	81.9	61.2	70.7	97.0
	e, dried after 27 hrs., 50°	87.5	51.1	85.1	64.1	80.8	105.0
	f, dried after 24 hrs., sun	58.0	15.1	71.6	29.0	66.7	101.0
E-3, fresh latex plus 0.1% thymol	*a*, dried immed. 70°	90.2	32.1	81.3	46.6	98.0	101.0
	b, dried after 3 hrs., 50°	88.5	29.8	78.7	42.2	76.8	82.8
	d, dried after 24 hrs., 70°	82.3	56.4	84.5	68.1	70.7	97.0
	e, dried after 27 hrs., 50°	85.6	52.5	84.5	64.1	80.8	90.9
	f, dried after 24 hrs., sun	55.7	19.7	72.4	29.6	76.8	94.9
E-4, fresh latex plus 0.2% thymol and 0.5% NaH SO_3	*a*, dried immed. 70°	123.0	80.0	85.1	66.4	104.0	90.9
	b, dried after 3 hrs., 50°	125.2	136.7	86.2	85.6	92.9	94.9
	d, dried after 24 hrs., 70°	116.4	129.5	84.8	86.8	86.9	99.0
	e, dried after 27 hrs., 50°	107.2	120.0	85.6	84.8	90.9	94.9
	f, dried after 24 hrs., sun	106.9	67.2	86.2	55.7	76.8	80.8
E-5, fresh latex plus 0.1% ea. of thymol & NaH SO_3	*a*, dried immed. 70°	121.3	13.1	86.8	23.3	74.7	60.6
	b, dried after 3 hrs., 50°	106.9	9.5	88.8	21.6	80.8	80.8
	d, dried after 24 hrs., 70°	109.8	72.8	87.1	56.3	97.0	94.9
	e, dried after 27 hrs., 50°	95.7	70.5	86.8	57.8	86.9	101.0
	f, dried after 24 hrs., sun	48.9	14.1	68.7	22.7	111.1	80.8
E-6, fresh latex plus 0.1% NaH SO_3	*a*, dried immed. 70°	109.2	15.4	86.2	25.0	80.8	80.8
	b, dried after 3 hrs., 50°	101.3	27.5	87.6	31.0	70.7	90.9
	d, dried after 24 hrs., 70°	99.3	66.2	85.1	60.3	90.9	84.8
	e, dried after 27 hrs., 50°	99.7	68.5	86.5	58.6	82.8	97.0
	f, dried after 24 hrs., sun	57.7	12.5	66.4	21.6	76.8	90.9
E-7, fresh latex plus 10% NaCl	*a*, dried immed. 70°	105.9	35.7	85.9	56.0	80.8	97.0
	b, dried after 3 hrs., 50°	89.5	34.4	85.3	55.7	76.8	97.0
	d, dried after 24 hrs., 70°	83.9	44.6	83.0	62.9	90.9	84.8
	e, dried after 27 hrs., 50°	93.4	29.2	84.2	45.7	86.9	84.8
	f, dried after 24 hrs., sun	58.4	14.8	73.6	36.5	80.8	94.9

* All activities are calculated on the basis of the dry papaya latex solids.

Milk clotting was determined as Units of Activity per gram.

Gelatin digestion was measured as milliequivalents of amino groups liberated from 20 ml. of 10 per cent gelatin solution by 100 mg. of dry papaya latex solids in two hours at 40°C.

Beef digestion was measured as ml. of beef digested per mg. of dry papaya latex solids. Beef powder (0.5 gm.) was digested with 0.5 mg. papain for 2 hours at 70°C.

clotting activities, except for the temporary rise noted in the latter. An entirely different picture is obtained from the beef-digestion activities,

FIGURE 1C. There does not seem to be any significant change on storage for approximately two years.

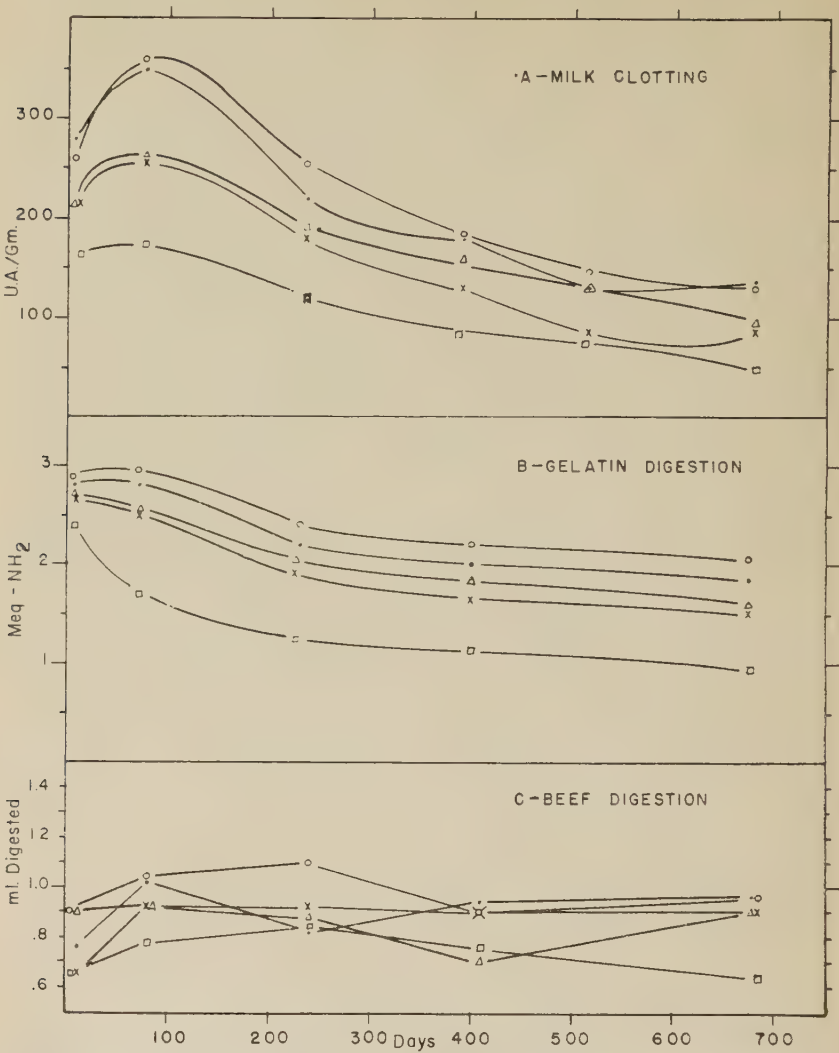

FIGURE 1. The effect of storage on the activity of papain prepared from fresh latex. Fresh latex dried at 70°C. (Sample E-1*a*, ○), at 50°C. (Sample E-1*b*, ●), 24-hour latex dried at 70°C. (Sample E-1*d*, ×), at 50°C. (Sample E-1*e*, △), and in the sun (Sample E-1*f*, □).

The effect of storage on the activities of the E-2 series (0.2 per cent thymol) is shown in FIGURE 2. The samples prepared by oven drying the 24-hour latex (E-2*d* and E-2*e*) unexpectedly exhibited a superior stability which is especially noticeable in the gelatin-digestion activities, FIGURE 2B. Possibly it should be noted, however, that the samples dried the first day, E-2*a* and E-2*b*, show a decreased stability, since the activities of E-2*d* and E-2*e* are

not much different from samples E-1*a* and E-1*b* prepared from untreated latex (FIGURE 1). Thymol (0.2 per cent) had no effect on the stability of the sun-dried sample. Reducing the thymol content made very little

FIGURE 2. The effect of storage on the activity of papain prepared from fresh latex treated with 0.2 per cent thymol. Freshly treated latex dried at 70°C. (Sample E-2*a*, ○), at 50°C. (Sample E-2*b*, ●), 24 hour-treated latex dried at 70°C. (Sample E-2*d*, ×), at 50°C. (Sample E-2*e*, △), and in the sun (Sample E-2*f*, □).

change in the stability of the resulting papains. The native activities of samples E-3*a* and E-3*b* (dried first day) again more nearly resembled those for the 24-hour controls (E-1*d* and E-1*e*), while the 24-hour samples E-3*d* and E-3*e* showed slightly better stability than the first-day controls. The

curves for the sun-dried sample E-3*f* were practically identical with those of the sun-dried control, E-1*f* (FIGURE 1).

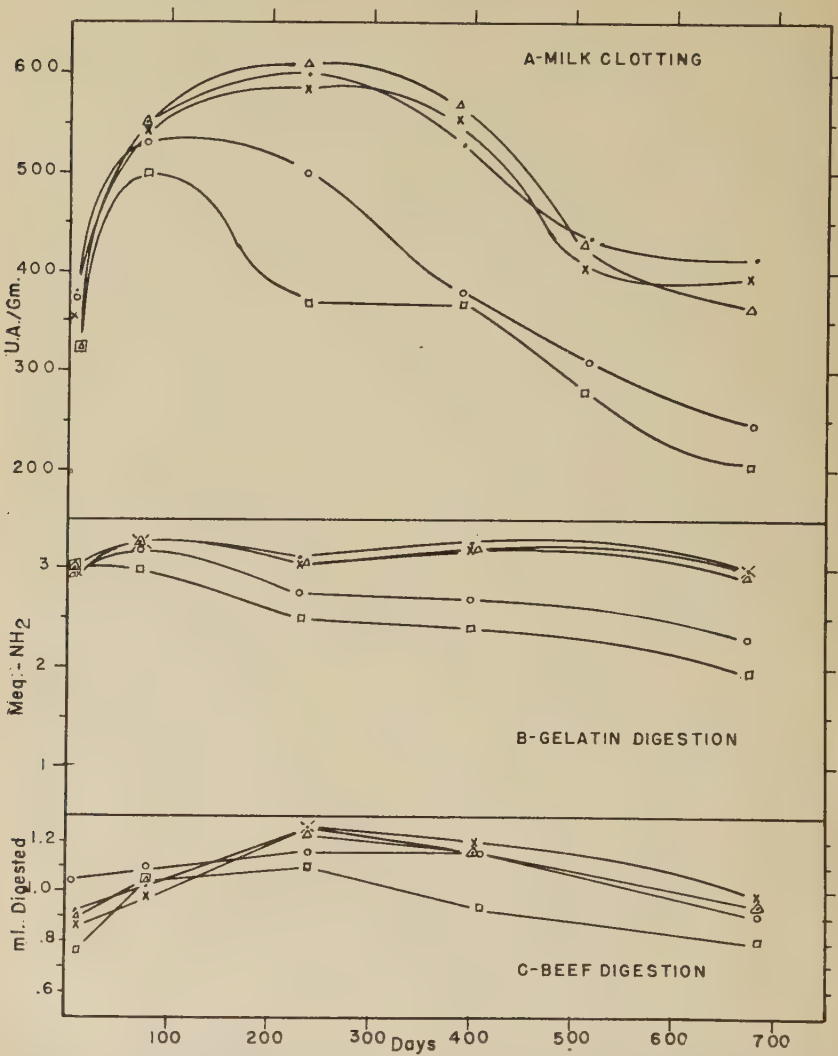

FIGURE 3. The effect of storage on the activity of papain prepared from fresh latex treated with 0.2 per cent thymol plus 0.5 per cent sodium bisulfite. Freshly treated latex dried at 70°C. (Sample E-4*a*, ○), at 50°C. (Sample E-4*b*, ●), 24 hour-treated latex dried at 70°C. (Sample E-4*d*, ×), at 50°C. (Sample E-4*e*, △), and in the sun (Sample E-4*f*, □).

The effect of storage on the activities of the E-4 series made up with 0.2 per cent thymol plus 0.5 per cent sodium bisulfite is shown in FIGURE 3. It is immediately apparent that the treatment given this series had a definite stabilizing and potentiating effect. Three of the samples, E-4*b*, E-4*d*, and E-4*e*, had a greater milk-clotting activity after 680 days of storage at room temperature than they had when originally prepared. The remaining two

samples were also quite active. Instead of the temporary rise in milk-clotting activity noted before, these three most active samples continued at the extremely high activity for more than a year before there was much decrease.

The picture for the gelatin-digestion activity is even more impressive. In all the other samples, there was a gradual decrease in gelatin-digestion activity with age. In this case, however, samples E-4*b*, E-4*d*, and E-4*e* showed no decrease in gelatin digestion after almost two years of storage. All of this series of samples were exceptional. The sun-dried sample, E-4*f*, had as great a milk-clotting and gelatin activity as did the best of the untreated samples. Even the beef-digestion activities were improved, showing very little if any of their usual erratic behavior.

A different stability picture was obtained, however, when the thymol and bisulfite content was reduced to 0.1 per cent in each sample (FIGURE 4). The native activities, A and B, of the first-day samples show that this treatment was detrimental and that their stability was of the same degree as has been generally found for sun-dried samples. The 24-hour samples, E-5*d* and E-5*e*, as with the E-2 and E-3 series, gave a stability pattern resembling that obtained with the first day controls, E-1*a* and E-1*b* (FIGURE 1).

The findings in the E-5 series of samples also apply to the E-6 (0.1 per cent bisulfite) series. The adverse effect of immediate drying, and the beneficial effect of allowing the reagent-latex mixture to stand at room temperature for 24 hours before drying on the stability of the native activity was again apparent.

The stability curves of the E-7 series (10 per cent sodium chloride) of samples are shown in FIGURE 5. These results, confirming those of Balls *et al.*,[6] show that sodium chloride has little if any effect on the stability of completely dried samples of papain.

Summary and Discussion

The effect of treating papaya latex with thymol, sodium bisulfite, and sodium chloride on the initial activity and stability of papain has been investigated. It has been shown that there is little if any difference between samples dried at 70° and 50°C. Sun drying, except in one instance, was markedly detrimental. Papain prepared from untreated latex that had been allowed to stand for 24 hours at room temperature prior to drying had a lower initial activity and was slightly less stable than papain prepared without the standing treatment.

The addition of 0.1 per cent thymol, 0.2 per cent thymol, 0.1 per cent sodium bisulfite, and 0.1 per cent thymol plus 0.1 per cent sodium bisulfite to fresh latex prior to drying produced almost identical results. Immediate oven drying gave material that, while it possessed good initial activity, was considerably less stable than untreated controls. When the treated latices were allowed to stand for 24 hours before oven-drying, the resulting papains were more stable than the best untreated controls.

The addition of 10 per cent of sodium chloride to the fresh latex had no marked effect on the stability of the resulting papain.

Very stable and potent papains were prepared by the addition of 0.2 per

cent of thymol plus 0.5 per cent of sodium bisulfite to the fresh latex. The stability was most marked when there was an interval between the chemical treatment of the latex and its drying. Three such samples showed no loss

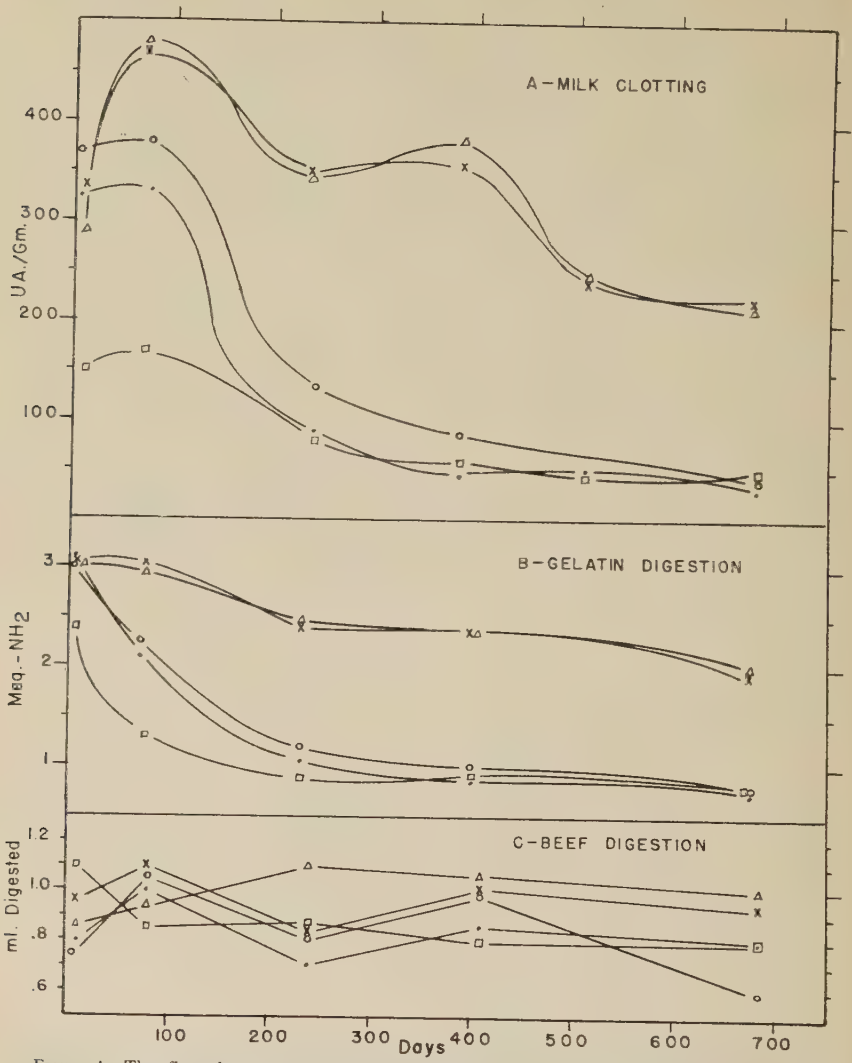

FIGURE 4. The effect of storage on the activity of papain prepared from fresh latex treated with 0.1 per cent thymol plus 0.1 per cent sodium bisulfite. Freshly treated latex dried at 70°C. (Sample E-5*a*, ○), at 50°C. (Sample E-5*b*, ●), 24 hour-treated latex dried at 70°C. (Sample E-5*d*, ×), at 50°C. (Sample E-5*e*, △), and in the sun (Sample E-5*f*, □).

of native activity by gelatin digestion after approximately two years of storage at room temperature.

It is apparent that both thymol and sodium bisulfite react with papaya latex and that this reaction is relatively slow. There is no indication

whether these chemicals react with the proteolytic enzymes *per se* or with inhibiting factors present in the latex. The stability of crystalline papain[7] would seem to favor the latter type of reaction.

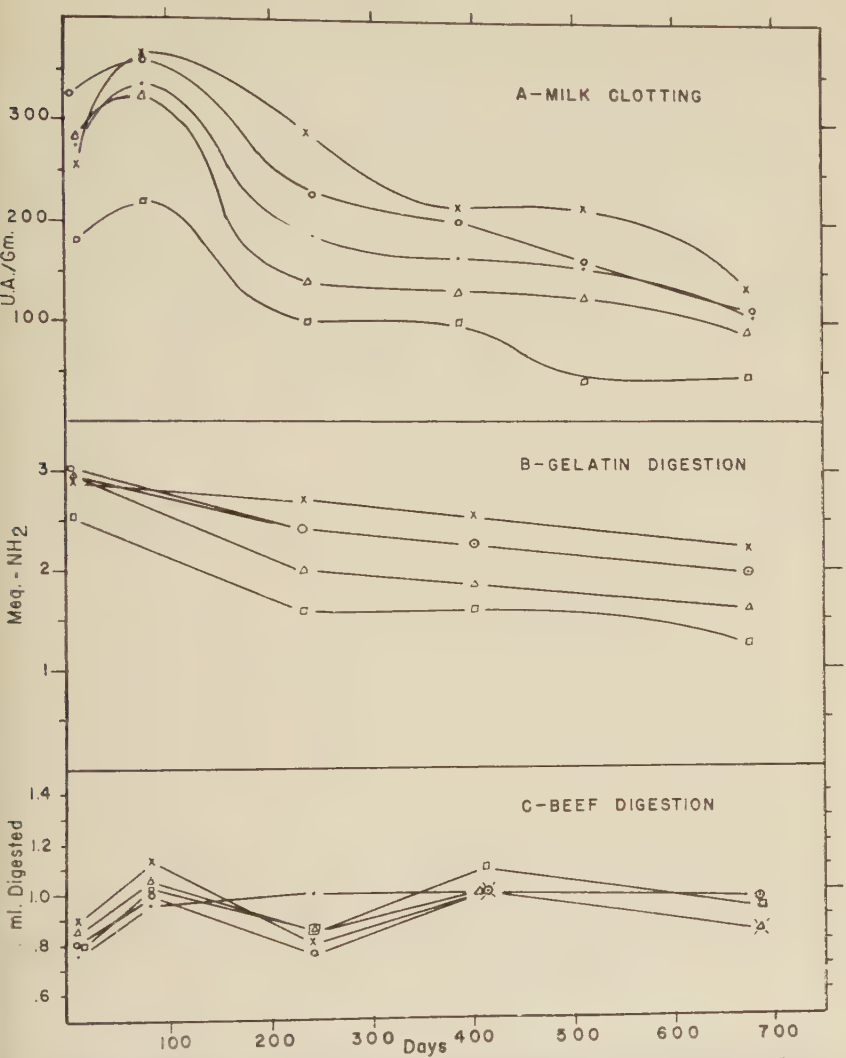

FIGURE 5. The effect of storage on the activity of papain prepared from fresh latex treated with ten per cent sodium chloride. Freshly treated latex dried at 70°C. (Sample E-7*a*, ○), at 50°C. (Sample E-7*b*, ●), 24 hour-treated latex dried at 70°C. (Sample E-7*d*, ×), at 50°C. (Sample E-7*e*,), △ and in the sun (Sample E-7*f*. □).

In the case of sodium bisulfite, it is at least possible that the stabilization is due to the antioxidant properties of the bisulfite. On the other hand, sodium thiosulfate[2] and sodium sulfide,[5] both reducing agents, have shown no such stabilizing action. The acidic reaction of sodium bisulfite might

possibly be involved, although citric acid produced no stabilization.[2] Balls *et al.*[6] have shown that the crystalline enzyme loses activity when mixed with latex. Inactivation also occurred when heated latex, having no activity itself, was substituted for fresh latex. This all points to a reaction of thymol and sodium bisulfite, with inhibiting factors present in fresh latex as the most probable explanation of their stabilizing action.

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DEMONSTRATION OF THE *IN VIVO* ACTIVITY OF PAPAIN

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The use of plant proteinases for the softening of meats has led to investigations concerning the effect of their ingestion upon animals and man. Tashiro and Schmidt[1] reported they were unable to discern any adverse effect in animals due to the feeding of papain-containing extracts. In fact, they suggested that "the optimum conditions of peptic digestion destroy papain." Lineweaver and Schwimmer[2] indicated accord with this view by suggesting that "it is doubtful if it (papain) will survive the acidity and other destructive agents that may be in gastric juice."

General interest[3–9] in the oral administration of papain, however, indicated the desirability of determining whether papain increased the digestion of protein *in vivo*. In this report, therefore, are given the results of studies which demonstrate that such activity does in fact exist.

Experimental

Papain Source Material. The source of papain for these studies was commercial papain (dried papaya latex). It had 163 units per gram by the milk-clotting test before activation and 212 units after activation.[3] The heat-inactivated material referred to below was prepared by autoclaving a portion of the papain at 15 lbs. pressure for 30 minutes. After autoclaving, it was dried at 60°C. under vacuum and ground to a fine powder. Its lack of proteolytic activity was confirmed by incubation with gelatin under standard test conditions.[3]

Procedure. Young adult rats in individual cages were starved for 24 hours and were then offered the following food mixture:

Casein	0.95 gram
Papain, unheated or heat-inactivated	0.05 gram
Glucose	0.33 gram

The rats used for test were those which voluntarily consumed the mixture within 7 to 15 minutes. They were sacrificed 2.5 hours after being fed the mixture. At that time, the stomach and fore-stomach contents were washed into a beaker with a small amount (25 ml.) of distilled water. The pH of the water suspension was noted and then adjusted to pH 4.85 to precipitate the proteins. The suspension was centrifuged and the clear or slightly cloudy supernatant fluid was poured off. The supernatant fluid was adjusted to pH 6.0 with 0.1 N sodium hydroxide, and a calculated amount of formalin was added so as to yield a formaldehyde concentration of about 9 per cent.[10] The solution was then titrated with 0.1 N sodium hydroxide to pH 9.0 as a measure of the protein digestion products.

Results and Discussion

A. Tests at the Natural pH of the Stomach Contents. The results (means ± s.e.) of the studies are summarized in TABLE 1.

It is seen clearly in TABLE 1 that marked differences resulted from the titrations of stomach contents. Thus, after the addition of formalin, 3.56 ± 0.12 ml. of 0.1 N sodium hydroxide were required to increase the pH from 6.0 to 9.0 for the stomach and fore-stomach contents of the rats fed papain. Only 1.97 ± 0.09 ml. of 0.1 N sodium hydroxide were required, however, for the comparable fraction from the control group of rats fed the heat-inactivated papain. The significance of the difference obtained was confirmed by statistical treatment of the data which showed P = <0.0001 or, that the result would be a chance occurrence less than once in ten thousand times. Thus, the appearance of the alkali titratable groups indicates that the proteolytic activity of papain increased the essentially

TABLE 1
EFFECT OF PAPAIN IN AIDING PROTEIN DIGESTION IN RATS

Index	*Test group fed active papain*	*Control group fed inactivated papain*
No. Rats	20	21
Males	12	12
Females	8	9
Weights, grams	206 ± 7	204 ± 10
Time to consume food, min.	10.0 ± 0.66	10.5 ± 0.86
pH stomach contents	3.89 ± 0.06	3.61 ± 0.07
	p = 0.003	
0.1 N sodium hydroxide to pH 4.85, ml.	1.56 ± 0.12	1.57 ± 0.096
Volume of centrifuge solids, ml.	1.66 ± 0.069	1.71 ± 0.068
0.1 N sodium hydroxide to pH 6.0, ml.	0.70 ± 0.079	0.49 ± 0.11
0.1 N sodium hydroxide to pH 9.0, ml.	3.56 ± 0.12	1.97 ± 0.092
	p = <0.0001	

normal rate of digestion 1.8 fold. Under less favorable conditions, this might conceivably be even greater. It is thus clear that papain administered orally contributes to the digestion of proteins in the stomach.

In harmony with the data which demonstrate that papain aided protein digestion in the stomach is the observation that the pH of the stomach contents was slightly higher (pH 3.89 ± 0.06) in the presence of papain than when heat-inactivated papain was fed (pH 3.61 ± 0.07). Statistically, the difference was indicated to be significant, P = 0.003, in that it would occur by chance only three times in a thousand. This agrees with the known anti-acid properties of protein hydrolysis products. This indication of an increased content of hydrolysis products in the rats fed papain over that noted in the control group was in harmony with the results obtained by titration of the stomach contents discussed above.

B. Tests in Conjunction with Anti-Acids. The foregoing results demonstrated clearly that papain was active at the natural pH of the stomach. It was of interest also to determine whether a decrease in gastric acidity

would enable papain to exert a greater effect and thereby indicate, indirectly, that papain was interfered with by the low pH of the gastric contents or by other possible inhibitors as suggested by Lineweaver and Schwimmer.[2] Accordingly, this was put to test by feeding anti-acids along with the papain-containing test meal. Three anti-acid combinations were chosen for this purpose: (1) aluminum hydroxide, (2) sodium bicarbonate, and (3) a combination of anti-acid agents, as supplied by "Al-Caroid" powder, including sodium bicarbonate, calcium carbonate, bismuth subcarbonate, magnesium carbonate, and magnesium oxide. The amounts of anti-acids fed and the results (means ± s.e.) obtained are summarized in TABLE 2.

TABLE 2

EFFECT OF PAPAIN IN AIDING PROTEIN DIGESTION IN RATS GIVEN ANTI-ACIDS

Index	*Sodium bicarbonate 73 mg. per rat*		*Aluminum hydroxide 25 mg. per rat*		*Al-Caroid (inactivated) 73 mg. per rat*	
	Control group fed inactivated papain	*Test group fed papain*	*Control group fed inactivated papain*	*Test group fed papain*	*Control group fed inactivated papain*	*Test group fed papain*
No. Rats	18	17	13	11	16	15
Males	15	17	8	8	15	15
Females	3	0	5	3	1	0
Weights, grams	162 ±4	172 ±6	159 ±11	184 ±8	180 ±7	191 ±9
Time to consume food, min.	14.1 ±1.3	14.9 ±1.3	11.8 ± 1.1	13.6 ±1.3	13.8 ±1.0	12.0 ±1.0
pH stomach contents	5.13±0.05	4.77±0.06	4.05± 0.10	3.97 ±0.17	5.03±0.06	5.07±0.10
Vol. of centrifuge solids, ml.	1.34±0.07	0.94±0.05	1.76± 0.08	1.32 ±0.094	1.44±0.08	0.96±0.08
	p = <0.0001		p = 0.0014		p = 0.00015	
0.1 N Sodium hydroxide to pH 6.0, ml.	0.45±0.02	0.50±0.05	0.25± 0.017	0.445±0.034	0.35±0.02	0.55±0.08
0.1 N Sodium hydroxide to pH 9.0, ml.	2.25±0.12	3.36±0.03	1.40± 0.04	2.90 ±0.17	2.00±0.03	3.72±0.43
	p = 0.0007		p = <0.0001		p = 0.0005	

In the presence of each of the anti-acids listed in TABLE 2, it is clearly evident that the ingestion of papain significantly increased the extent to which the casein had been digested. The significance of the differences was confirmed by statistical treatment of the data, which demonstrated that in all cases P was less than 0.001, a criterion usually accepted as highly significant.

The increased amount of protein digestion, due to the addition of papain to the protein test meal, varied somewhat from test to test. When aluminum hydroxide was added to the test meal, about 2.5 times as much protein digestion occurred in the group receiving papain as occurred in the test control group. Upon the addition of sodium bicarbonate to the test meal, about 1.5 times as much protein digestion resulted in the group receiving papain as was noted in the control animals. In the third instance, in which Al-Caroid anti-acids were employed, about 1.9 times as much protein digestion was observed when papain was included with the test meal as

occurred in the control rats. A comparison of these ratios with that which resulted (1.8) from feeding papain without an added anti-acid makes it evident that the anti-acids neither increased nor decreased the effectiveness of the papain in the stomach. Thus, no evidence was obtained to substantiate the view that the stomach contents would destroy papain activity because of gastric acidity, or the lack of it, or other adverse environmental factors present in the stomach.

In the presence of each of the three anti-acids, the volume of solids in the stomach after centrifuging was less in the presence of papain than when heat-inactivated papain was fed. In each case, the differences are statistically significant. The results are in the same direction as the formol titration data and can be interpreted only as confirming the *in vivo* activity of the papain in digesting the protein of the test meal.

Thus, from the evidence presented here, it is clear that *in vivo* papain activity, as supplied by average dried papaya latex preparations, is easily and convincingly demonstrated by studies on the extent of intragastric digestion.

Summary

1. Studies with rats are described in which the extent of *in vivo* gastric protein digestion was followed by examination of the stomach and forestomach contents 2.5 hours after the ingestion of a test meal.

2. From the results of formol titrations of the gastric contents and measurements of the volumes of undigested residues, a 1.5- to 2.1-fold greater protein digestion, produced by feeding papain, was clearly demonstrated.

3. The presence of anti-acids did not modify the effect of the papain on digestion.

4. On the basis of these results, it was concluded that orally ingested papain has *in vivo* digestive activity. No evidence was seen that the gastric contents adversely affect the digestive activity of the enzyme.

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THE EFFECT OF THE ACTIVITY STATE OF THE ENZYMES ON THE *IN VIVO* ACTIVITY OF PAPAIN

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In earlier papers,[1,2] *in vitro* methods for the estimation of the activity of the proteolytic enzymes of papaya latex were compared. As pointed out there, these proteolytic enzymes exist in two activity states, the native or active state and the reversibly inactive state. The former digests proteins and clots milk without prior activation, whereas the latter exhibits its proteolytic properties only after activation by such agents as HCN, H_2S, mercaptans, cysteine, *etc.* It has been shown, however, that certain proteinaceous substrates, particularly beef[1–3] and heat-coagulated egg albumin,[4] have the ability to activate this inactive form during *in vitro* proteolysis.

Recently, Arnold, Schmitz, and Blumberg[5] have shown that papain, when added to a high protein test meal, significantly increased the extent of protein digestion occurring in the rat's stomach. The papain these investigators fed had approximately equal quantities of the enzymes in each of the two activity states. Additional investigations have therefore been carried out concerning the digestive power of these two activity states of the enzymes *in vivo*.

The activity of two papain samples having widely different ratios of active-to-reversibly-inactive enzymes were compared to determine the extent of intragastric digestion, and, more particularly, whether the reversibly inactive forms were active *in vivo*. Casein was chosen as the protein for the test meal, since previous investigations[1] had shown that it is not acted upon *in vitro* by the reversibly inactive enzymes. Beef or heat-coagulated fresh egg albumin could not be used for this test, since their *in vitro* activating effect might mask any *in vivo* activation.

Experimental

A. Comparison in vitro. A papain preparation, Papain A, with the enzyme content largely in the active state, was prepared from frozen fresh papaya latex by treatment with 0.5 per cent sodium bisulfite prior to drying, as has been described elsewhere.[6,7] National Formulary Reference papain was used as the low active, high reversibly inactive sample. A sample of commercial papain was irreversibly inactivated by autoclaving for 30 minutes at 15 lbs. to be used as a negative control. These three samples were tested by the Blau modification[1] of the Balls and Hoover[8] milk-clotting method, both with and without HCN activation. They were also tested by an egg digestion method[4] and a gelatin digestion-formol titration procedure.[1] The results are given in TABLE 1.

It may be seen that there is a vast difference between the native (not activated) activities of the two active samples. The ratios of the native activities and the total (native plus reversibly inactive) activities of the N. F. Reference papain and Papain A are 1:75 and 1:3.9 respectively, as measured by the milk-clotting procedure. The egg digestion method gives

a slightly lower ratio for the total activities, 1:1.5. It is apparent that if *in vivo* digestion is produced also by the reversibly inactive form of the enzymes, these two active preparations should show it quite clearly.

B. Comparison in vivo. Rats of about 200 gm. body weight were starved for 24 hours and then given a high protein test meal consisting of 1000 mg. of casein, 330 mg. of dextrose and the desired amount of papain added at the expense of the casein. Those animals that ate their test meal within 20 minutes were sacrificed two hours from the time the test meal was offered. At that time, their stomachs were removed and the contents washed into a suitable container. This suspension was adjusted to pH 4.85 with 0.1N NaOH and then centrifuged. The clear supernatant liquid was decanted and adjusted to pH 6.0 with additional 0.1N NaOH. Ten milliliters of freshly neutralized 40 per cent formaldehyde solution were then added and the solution titrated to pH 9.0 with 0.1N NaOH. The volume of alkali

TABLE 1

THE *In Vitro* ACTIVITY OF THREE PAPAINS

Sample	*Milk clotting U.A./gm.**		*Egg digestion, relative activity*	*Gelatin digestion, mEq.-NH_2†*
	HCN activated	*Not activated*		
Papain A	361	361	1.52	3.14
NF reference papain	92.3	4.8	1.0	0.23
Autoclaved papain	0.0	0.0	0.0	0.0

* Units of Activity per gram.

† Milliequivalents of amino groups liberated by 100 mg. of unactivated papain digesting 20 ml. of citrate buffered (pH 5), 10 per cent gelatin solution for 2 hours at 40°C.

used in the last titration was a measure of the amount of amino groups present and, consequently, of the extent of protein digestion.[9]

For the first experiment, 50 mg. of the papain preparations were included in the test meals. The rats refused, however, to eat those containing this much Papain A. In the next experiment, 20 mg. of papain were used, but there was too much digestion in the Papain A group and a considerable portion of the digested material had left the stomach in the two-hour period. In two cases, the stomachs were practically empty. For the subsequent experiments, more nearly threshold doses of 1.25 mg. and 5 mg. of Papain A and 5 mg. and 20 mg. of N. F. Reference and autoclaved papains were fed.

Results

The results of the *in vivo* trials are given in TABLE 2. The autoclaved and completely inactive papain was used as a negative control to measure the amount of spontaneous digestion under the conditions. That it was completely inactive was shown by the lack of increase of digestion between the 5 mg. and 20 mg. doses respectively of this preparation. It may be seen that feeding active papain with the test meal materially increased the amount of digestion of the protein. This increase ranged from 34 to 101 per cent, depending on the dose of the papain given. The amount of digestion increased with the dose of papain in a regular manner. These

results, therefore, were clear confirmation that papain taken orally could increase significantly the amount of gastric digestion of protein.[5]

Since the values obtained with the 5 mg. and 20 mg. quantities of autoclaved papain are identical within the limits of variation of the method,

TABLE 2

In Vivo ACTIVITY OF PAPAIN AS MILLIEQUIVALENTS × 10 OF AMINO GROUPS LIBERATED DURING TWO HOURS' DIGESTION OF CASEIN IN THE RAT STOMACH

	Papain A		*N. F. Reference papain*		*Autoclaved papain*	
Dose	1.25 mg.	5 mg.	5 mg.	20 mg.	5 mg.	20 mg.
Number of animals	14	24	27	11	26	9
Mean	1.34	1.87	1.50	2.01	0.99	1.04
Standard errors of the mean	±0.048	±0.077	±0.070	±0.156	±0.040	±0.073

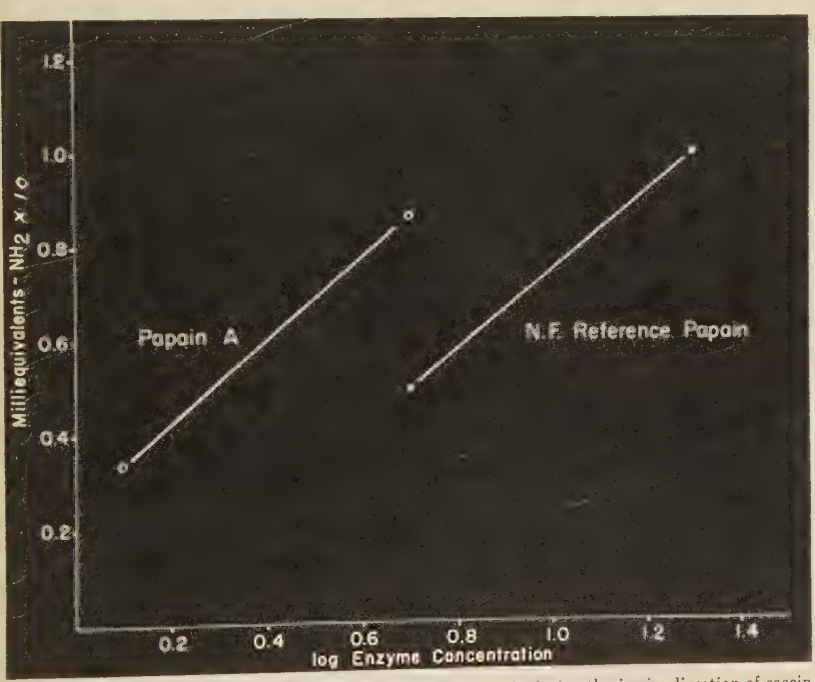

FIGURE 1. The average gain in amino groups liberated by papain during the *in vivo* digestion of casein in the rat's stomach.

they may be combined to give a control blank value for the other experiments. This yields an average control figure of 0.10 milliequivalents of liberated amino groups, which represents the amount of normal digestion. Subtraction of this value from the averages in TABLE 2 gives the average gains in liberated amino groups produced by the respective quantities of papain. These results are shown graphically in FIGURE 1.

Since the curves are substantially parallel, the ratio of activity of the two

samples may be readily calculated from them. The average ratio of *in vivo* potency of Papain A to the N. F. Reference papain, as well as the calculated ratios between the *in vitro* native and total activities of these samples are given in TABLE 3.

It is readily apparent that the *in vivo* ratio of activities of these papains agrees well with the *in vitro* activated milk-clotting value and with the egg-digestion figure, which is a spontaneously activated value. The native milk-clotting value, which does not measure the reversibly inactivated enzymes, widely diverges from the *in vivo* results. These ratios indicate that the reversibly inactive form of the enzymes contributes to the *in vivo* digestion of protein in substantially full measure.

It will also be noted in TABLE 3 that, although the *in vivo*, the activated milk-clotting, and the egg-digestion ratios of activity are of the same magnitude, there are apparently significant differences between them. The full significance of this is not clear, but it probably is connected with the activation phenomenon. Some indications for this are to be found in the literature on the activation of papain.[10, 11] Both Fruton and Bergmann[10] and Scott

TABLE 3
THE RELATIVE *In Vivo* AND *In Vitro* POTENCIES OF PAPAIN A AND N.F. REFERENCE PAPAIN

Test method	*Ratio of Papain A to N.F. Reference papain*
Rat, *in vivo*	2.7:1
Native, milk clotting	75:1
Activated, milk clotting	3.9:1
Egg digestion	1.5:1

and Sandstrom[11] have reported an increased activation of papain with thiols (H_2S, cysteine, or mercaptans) as compared with HCN. The Papain A used in our work contained none of the reversibly inactive enzymes and was therefore insensitive to activators. On the other hand, the N. F. Reference papain contains a high proportion of this form of the enzymes and is therefore amenable to activation. It may be that the activation of the N. F. Reference papain during the proteolysis of heat-denatured fresh egg albumin was due to either the original presence of, or the liberation of, thiols in the substrate. Thus, it would appear that the differences in the *in vitro* activity ratios resulted from differences in the activators involved. If this is the mechanism, then the *in vivo* activation can be assumed to be produced by an activator or activators having activating properties ranging between those of HCN and those in the egg albumin substrate. In any event, the data demonstrates that the reversible form of the enzyme contributes markedly to the digestive power *in vivo*.

Summary

The potency of the proteolytic enzymes of papaya latex for digestion of protein in the stomach has been examined by studies with rats. The extent

of digestion was estimated by formol titration of the stomach contents after test meals of casein plus various doses of papain preparations. Addition of papain to the test meal significantly increased the amount of gastric digestion.

A comparison of the activities of two preparations containing markedly different amounts of reversibly inactive enzymes demonstrated that this form of the enzymes, as well as the active form, is effective *in vivo*. From the results, it is evident that papain can facilitate the digestion of protein when taken orally, and that its *in vivo* activity is proportional to the total (active plus reversibly inactive) enzyme rather than to only the naturally active enzyme content.

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THE *IN VIVO* PROTEOLYTIC ACTIVITY OF PAPAIN AS DEMONSTRATED BY NITROGEN BALANCE STUDIES IN DOGS

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In earlier papers[1, 2] in this series, it was shown that activity of orally ingested papain could be demonstrated *in vivo* in rats. This disproved earlier suggestions[3, 4] that papain could not be expected to withstand gastric acidity and peptic digestion. To determine, further, how readily papain activity *in vivo* could be demonstrated in other animals, dog-feeding studies were instituted in which the effect of adding papain to soy bean meal diets was followed by nitrogen balance determinations. The results of these studies are given in this paper.

Experimental

Dogs. The adult mongrel dogs selected, some of whom had been used satisfactorily in previous feeding experiments, were of moderate size and parasite free. Their quarters were air conditioned and maintained at constant temperature. For these studies, the dogs were housed in metabolism cages which had sheet metal sides and wide mesh screen bottoms. There were three urine collection periods weekly, two 2-day samples during the week and a 3-day sample over the weekend. The completeness of urine collections was verified for each collection period by creatinine determinations. Nitrogen analyses were made by the macro-Kjeldahl procedure with a copper sulfate catalyst.

As in a previous publication from this laboratory,[5] the daily caloric needs of the dogs were based on the optimum body weight at a nutritive state of 0.3. This weight was estimated for each dog by the Cowgill and Drabkin[6] formula $W = (0.3L)^3$, where W = weight in kilograms and L = length in centimeters. The caloric allotments were subsequently fixed between 60 and 80 calories per kilogram for each dog, to minimize weight fluctuations. The experimental findings, however, have been expressed in terms of the actual weights of the animals rather than upon their calculated optimum weights.

Papain. The dried papain used in these studies was prepared from papaya latex.† This "active" papain contained approximately 460 milk-clotting units[7] per gram. The control diets containing an "inactive" papain were made using a dried, autoclaved (120°C. for 30 minutes) sample which no longer possessed any proteolytic activity. The papains were added to the diets in the amount of one per cent of the protein content of the ration. This constituted roughly 0.2 per cent of the diet or 250–400 mg. of papain powder per dog per day, dependent upon the weights of the dogs.

Soy Bean Meal. Unheated soy bean meal was selected as the protein

* The technical assistance of Daniel J. Bradley, Jr. is gratefully acknowledged.
† The papain used in these studies was supplied through the courtesy of E. T. Hinkel, Jr.

source because it is known to be incompletely digested. Two samples of soy bean meal were fed during the course of these studies. The first had been prepared earlier in this laboratory by ether extracting ground raw soy beans at room temperature. This sample contained 6.66 per cent nitrogen. The other was represented by the manufacturer to be unheated, defatted soy bean grits.* It contained 8.48 per cent nitrogen.

Procedure. The studies consisted of a comparison of the amounts of soy bean protein digested by dogs given active or inactive papain in the diet. The metabolic nitrogen values required for calculating percentage digestibility were based on the fecal nitrogens which resulted from the feeding of lactalbumin,† an essentially completely digestible protein. For calculations of protein biological values, estimations of the endogenous nitrogens were also required. These were based on the urinary nitrogen excretion during the same lactalbumin feeding periods.

The dogs were brought into comparable physiological states by equilibrating them at minimal nitrogen intakes. The method is well established from the studies of several investigators.[8, 9] It was accomplished by maintaining the dogs on a nitrogen-free diet, described earlier by Kade *et al.*,[10] supplemented with an amount of lactalbumin which would permit the dogs to come into nitrogen equilibrium at a low level of nitrogen intake.

When the dogs had been brought near or into nitrogen equilibrium, they were fed the unheated, defatted soy bean meal- and papain-containing diets. The basal nitrogen-free diet to which the test materials were added was formulated to supply only a small amount of fat so that it would tend to pass more rapidly through the stomach upon ingestion and thus serve to minimize the action of pepsin. It had the following composition: glucose (Cerelose) 46, dextrin 24.5, hydrogenated vegetable oil (Primex) 3, salts (Jones and Foster[11] 4, methyl cellulose 2.5, cod liver oil (2,000 units vitamin A, 250 units vitamin D per gram) 0.75, wheat germ oil (Fyvee) 0.25, liver extract (Wilson's fraction 0) 0.2, choline chloride 0.3, niacin 0.006, thiamine hydrochloride 0.0006, calcium pantothenate 0.0006, riboflavin 0.0006, pyridoxine hydrochloride 0.0006, and folic acid 0.0002. The diet supplied 3.83 calories per gram.

During the course of the study, the dogs were usually fed the diet containing either active or inactive papain for two or three weeks before they were fed the alternate experimental diet. At the end of this experimental period, they were re-equilibrated on the lactalbumin containing diet. This was done to re-establish the fact that they were at or near nitrogen equilibrium and to redetermine their metabolic and endogenous nitrogen excretion.

It became evident early in the course of the study that active papain in the diet improved protein digestion, presumably making more peptides and amino acids available to the body. Supplements of sulfur amino acids, therefore, at one per cent of the amount of soy bean protein, were subsequently added to some of the diets. This was done, in part, to permit

* Obtained from A. E. Staley Manufacturing Co. under the designation I-8-20.
† Borden's lactalbumin 15-42, 12.6 per cent nitrogen.

bringing the dogs into nitrogen equilibrium at lower intake levels of soy bean protein, since it is known that soy bean protein is improved by the addition of cystine and/or methionine. In part, the amino acids were added to determine their quantitative effect on the utilization of the protein in the presence of active and inactive papain.

Results and Discussion

In FIGURE 1 are shown the changes in digestibility and in the calculated amount of nitrogen required to maintain dogs in nitrogen equilibrium resulting from the addition of active or inactive papain to soy bean meal-containing diets.

During the first seven weeks of the study, dogs 55, 62, and 66 received the laboratory extracted soy bean meal. Henceforth, all dogs were fed the commercially prepared material. In all calculations, no distinction has been made between the use of the two soy bean meals, since there was no evidence that there was any material difference in the results obtained under each of the feeding trials.

Effect of Papain on Digestibility. The extent to which the unheated, defatted soy bean protein was digested by the dogs was calculated by the formula[12]:

$$\text{Digestibility in per cent} = \frac{\text{Food N} - (\text{Fecal N} - \text{Metabolic N})}{\text{Food N}} \times 100.$$

As indicated above, the metabolic nitrogen figure was based on the lact-albumin fecal nitrogen observed at the time the dogs were at or near nitrogen equilibrium. This more nearly represents the metabolic nitrogen than does the value obtained by feeding a nitrogen-free diet, as is sometimes done, since there is no additional demand on the tissues for nitrogen, as is the case when a nitrogen-free diet is fed. A single metabolic nitrogen figure was used throughout for each dog based on the average of all the values obtained for that dog.

It may be seen from FIGURE 1 and from the data in TABLE 1 that amino acid supplementation had little effect on digestibility, as might have been anticipated. Accordingly, single digestibility figures for active and for inactive papain were calculated for each dog and are summarized in TABLE 2.

It may be seen from the summary data in TABLE 2 that the presence of active papain in the diet was responsible for greater digestion of soy bean protein in all the dogs. In the case of six out of the nine dogs, the differences were beyond any probable limit of spontaneous variation ($P = 0.006$ or less).* Since these dogs did not have impaired digestive functions and

* The P values were derived from the corresponding t values, taking into account the number of weeks of test utilized in calculating the means and where

$$t = \frac{x_1 - \bar{x}_2}{\sqrt{se^2_{\bar{x}_1} + se^2_{\bar{x}_2}}}.$$

Increased significance could have been ascribed to the means by basing the calculations on the 2- and 3-day collection periods. It was believed, however, that the more conservative procedure employed here, that of basing the calculations on the results of a week's collections, would be more meaningful where it was observed to lead to significant differences.

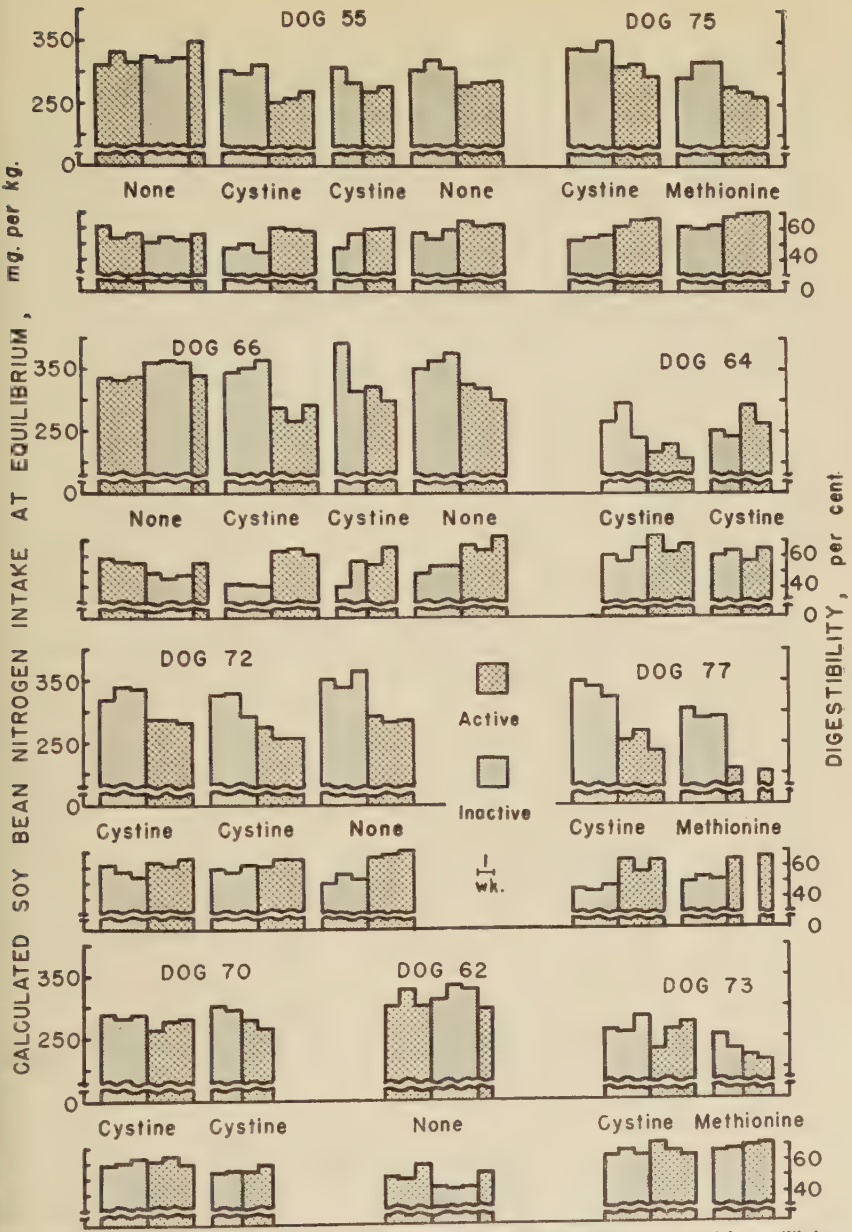

FIGURE 1. Graphic representation of the calculated soy bean nitrogen intakes required for equilibrium, together with the corresponding percentage digestibility values. The amino acid supplements, where added, are indicated between the histograms. A one-week break which occurred during the feeding of active papain to dog 77 is due to a lost (spilled) feces sample.

had not been subjected to any surgical procedures, the fact that active papain increased the digestion of protein from 54.7 per cent to 63.1 per cent, an increased utilization of 15.5 per cent, was still more noteworthy.

TABLE 1

WEEKLY NITROGEN BALANCE DATA OF DOGS FED LACTALBUMIN OR SOY BEAN MEAL DIETS*

Dog no.	Weight	Nitrogen source	Nitrogen intake		Total N-intake	Amino acid supplement	Caroid	N-excretion		Nitrogen balance	Calculated N-intake at equilibrium	Digestibility
			test protein	basal diet				urinary	fecal			
	kg.		mg./kg./day		mg./day			mg./day		mg./kg./day	mg./kg./day	%
55	7.0	SBM	300	6	2140	none	active	1208	1020	−13	313	61.8
	6.9	SBM	304	6	2140	none	active	1164	1175	−29	333	54.6
	6.9	SBM	304	6	2140	none	active	1133	1095	−13	317	58.3
	6.9	SBM	304	6	2140	none	inactive	1055	1234	−22	326	51.8
	6.8	SBM	309	6	2140	none	inactive	1065	1148	−11	320	55.8
	6.8	SBM	309	6	2140	none	inactive	1055	1197	−16	325	53.6
	6.7	SBM	313	6	2140	none	active	1202	1123	−28	351	57.0
	7.2	Lact.	88	6	674			661	182	−23	111	
	7.2	Lact.	88	6	674			545	183	−8	96	
	7.2	SBM	330	6	2416	l-cystine	inactive	765	1469	+25	305	47.6
	7.2	SBM	330	6	2416	l-cystine	inactive	793	1403	+31	299	50.3
	7.2	SBM	330	6	2416	l-cystine	inactive	785	1519	+16	314	45.5
	7.2	SBM	292	6	2145	l-cystine	active	840	1037	+37	255	61.1
	7.2	SBM	292	6	2145	l-cystine	active	861	1052	+32	260	60.4
	7.1	SBM	297	6	2145	l-cystine	active	886	1082	+25	272	59.0
	6.8	Lact.	93	7	683			511	245	−11	104	
	6.8	Lact.	93	7	683			472	197	+2	91	
	6.8	SBM	329	6	2282	l-cystine	inactive	763	1405	+17	312	47.3
	6.8	SBM	329	6	2282	l-cystine	inactive	809	1170	+45	284	57.6
	6.9	SBM	325	6	2282	l-cystine	active	799	1110	+54	271	60.3
	6.9	SBM	325	6	2282	l-cystine	active	851	1105	+47	278	60.5

	6.9	Lact.	91	7	683			559	207	−12	103	
	6.9	Lact.	91	7	683			480	199	+1	90	
	6.9	SBM	315	7	2225	none	inactive	1014	1126	+12	303	58.5
	6.9	SBM	315	7	2225	none	inactive	1059	1217	−7	322	54.4
	6.9	SBM	315	7	2225	none	inactive	1094	1095	+5	310	59.9
	6.8	SBM	297	8	2073	none	active	1043	906	+18	279	66.1
	6.8	SBM	297	8	2073	none	active	1020	966	+13	284	63.2
	6.8	SBM	297	8	2073	none	active	1041	959	+11	286	63.5
	6.7	Lact.	95	7	683			538	180	−7	102	
	6.8	Lact.	93	7	683			464	228	−1	94	
62	11.5	SBM	300	6	3513	none	active	1507	2047	−4	304	51.9
	11.4	SBM	303	6	3513	none	active	1690	2105	−25	328	50.3
	11.2	SBM	308	6	3513	none	active	1675	1781	+5	303	59.5
	11.2	SBM	308	6	3513	none	inactive	1312	2287	−8	316	45.1
	10.9	SBM	317	6	3513	none	inactive	1353	2341	−17	334	43.6
	10.8	SBM	319	6	3513	none	inactive	1320	2304	−10	329	44.6
	10.7	SBM	322	6	3513	none	active	1292	1967	+24	298	54.2
	11.3	Lact.	92	7	1120			774	359	−1	93	
64	9.9	Lact.	91	7	973			715	315	−6	97	
	10.0	SBM	340	7	3469	l-cystine	inactive	1103	1648	+72	268	60.1
	10.2	SBM	333	7	3469	l-cystine	inactive	1270	1800	+39	294	56.6
	10.2	SBM	275	7	2875	l-cystine	inactive	1207	1287	+37	238	65.5
	10.2	SBM	275	7	2875	l-cystine	active	1225	1072	+57	218	73.0
	10.3	SBM	233	8	2478	l-cystine	active	1198	1232	+5	228	62.2
	10.3	SBM	233	8	2478	l-cystine	active	1130	1099	+24	209	67.6
	10.3	Lact.	87	7	973			793	288	−10	97	
	10.3	Lact.	87	7	973			718	281	−3	90	

TABLE 1—*Continued*

Dog no.	*Weight*	*Nitrogen source*	*Nitrogen intake*		*Total N-intake*	*Amino acid supplement*	*Caroid*	*N-excretion*		*Nitrogen balance*	*Calculated N-intake at equilibrium*	*Digestibility*
			test protein	*basal diet*				*urinary*	*fecal*			
	kg.		*mg./kg./day*		*mg./day*			*mg./day*		*mg./kg./day*	*mg./kg./day*	*%*
	10.5	SBM	267	7	2875	l-cystine	inactive	1301	1436	+13	254	60.3
	10.7	SBM	262	7	2875	l-cystine	inactive	1327	1332	+20	242	63.9
	10.6	SBM	264	7	2875	l-cystine	active	1638	1522	−27	291	57.3
	10.7	SBM	262	7	2875	l-cystine	active	1621	1283	−3	265	65.6
73	7.3	Lact.	99	6	767			636	275	−20	119	
	7.3	SBM	307	8	2301	l-cystine	inactive	894	1103	+42	265	63.9
	7.2	SBM	311	8	2301	l-cystine	inactive	939	995	+51	260	68.6
	7.1	SBM	316	8	2301	l-cystine	inactive	1018	1072	+30	286	65.2
	7.1	SBM	270	9	1983	l-cystine	active	894	814	+39	231	72.7
	7.0	SBM	274	9	1983	l-cystine	active	1001	911	+10	264	67.8
	6.9	SBM	278	9	1983	l-cystine	active	984	975	+3	275	64.5
	6.9	Lact.	104	8	775			508	269	0	104	
	7.0	SBM	280	9	2024	dl-methion.	inactive	897	953	+25	255	66.4
	7.0	SBM	280	9	2024	dl-methion.	inactive	784	916	+46	234	68.2
	7.1	SBM	256	9	1880	dl-methion.	active	799	833	+35	221	70.2
	7.1	SBM	256	9	1880	dl-methion.	active	797	795	+41	215	72.2
	7.1	Lact.	102	8	775			530	321	−8	110	
66	9.8	SBM	306	6	3052	none	active	1773	1593	−32	338	59.3
	9.7	SBM	309	6	3052	none	active	1632	1653	−24	333	57.3
	9.6	SBM	312	6	3052	none	active	1640	1678	−28	340	56.5

9.4	SBM	319	6	3052	none	inactive	1574	1883	−43	362	49.8
9.4	SBM	319	6	3052	none	inactive	1503	1980	−46	365	46.6
9.3	SBM	322	6	3052	none	inactive	1487	1930	−39	361	48.2
9.3	SBM	322	6	3052	none	active	1538	1690	−19	341	56.1
9.8	Lact.	92	6	962			714	328	−8	100	
9.7	Lact.	93	6	962			671	396	−10	103	
9.8	SBM	347	7	3469	l-cystine	inactive	1129	2333	+1	346	42.8
9.8	SBM	347	7	3469	l-cystine	inactive	1178	2349	−6	353	42.4
9.7	SBM	350	7	3469	l-cystine	inactive	1221	2393	−15	365	41.1
9.8	SBM	347	7	3469	l-cystine	active	1255	1624	+60	287	63.3
9.9	SBM	343	7	3469	l-cystine	active	1192	1545	+74	269	65.6
10.0	SBM	340	7	3469	l-cystine	active	1316	1693	+46	294	61.3
9.7	Lact.	93	7	973			759	320	−11	104	
9.7	Lact.	93	7	973			655	387	−7	100	
9.7	SBM	372	7	3680	l-cystine	inactive	1296	2547	−17	389	40.3
9.8	SBM	368	7	3680	l-cystine	inactive	1133	2035	+52	316	57.5
9.9	SBM	364	7	3680	l-cystine	active	1251	2033	+40	324	54.3
10.0	SBM	361	7	3680	l-cystine	active	1215	1610	+86	275	65.8
9.9	Lact.	91	7	973			642	333	0	91	
10.0	Lact.	90	7	973			507	363	+10	80	
10.1	SBM	351	7	3622	none	inactive	1457	2176	−1	352	49.6
9.9	SBM	358	7	3622	none	inactive	1624	2024	−3	361	53.8
9.8	SBM	362	7	3622	none	inactive	1736	2024	−14	376	53.8
9.8	SBM	327	8	3271	none	active	1840	1421	+1	326	67.3
9.9	SBM	323	8	3271	none	active	1727	1522	+2	321	64.2
10.0	SBM	320	7	3271	none	active	1840	1244	+19	301	72.7
9.9	Lact.	91	7	973			763	308	−10	101	
10.0	Lact.	90	7	973			804	362	−19	109	

TABLE 1—*Continued*

Dog no.	Weight	Nitrogen source	Nitrogen intake: test protein	Nitrogen intake: basal diet	Total N-intake	Amino acid supplement	Caroid	N-excretion: urinary	N-excretion: fecal	Nitrogen balance	Calculated N-intake at equilibrium	Digestibility
	kg.		mg./kg./day		mg./day			mg./day		mg./kg./day	mg./kg./day	%
70	7.3	Lact.	81	5	625			805	199	−52	133	
	7.4	SBM	247	6	1863	l-cystine	inactive	1189	992	−43	290	60.0
	7.2	SBM	253	6	1863	l-cystine	inactive	1125	973	−33	286	61.0
	7.1	SBM	257	6	1863	l-cystine	inactive	1177	910	−32	289	64.4
	7.1	SBM	257	6			active					
	7.0	SBM	261	6	1863	l-cystine	active	937	962	−5	266	61.6
	6.8	SBM	268	6	1863	l-cystine	active	1037	894	−10	278	65.3
	6.8	SBM	268	6	1863	l-cystine	active	951	994	−12	280	59.9
	6.7	Lact.	88	6	625			459	245	−12	100	
	6.9	SBM	305	8	2155	l-cystine	inactive					
	6.8	SBM	310	8	2155	l-cystine	inactive	895	1221	+6	304	54.8
	6.9	SBM	305	8	2155	l-cystine	inactive	905	1200	+7	298	55.8
	7.0	SBM	287	8	2065	l-cystine	active	853	1175	+5	282	55.1
	7.0	SBM	287	8	2065	l-cystine	active	845	1086	+19	268	59.4
	6.9	Lact.	92	7	681			431	274	−3	95	
	7.0	Lact.	91	7	681			417	269	−1	92	
72	5.7	Lact.	111	8	681			613	184	−20	131	
	5.8	SBM	337	11	2016	l-cystine	inactive	861	1058	+17	320	61.5
	5.7	SBM	343	11	2016	l-cystine	inactive	876	1127	+2	341	58.1
	5.8	SBM	337	11	2016	l-cystine	inactive	845	1178	−1	338	55.6
	5.7	SBM	295	11	1744	l-cystine	active	782	919	+8	287	63.5
	5.8	SBM	290	11	1744	l-cystine	active	749	962	+6	284	61.0
	5.6	SBM	300	11	1744	l-cystine	active	776	870	+18	282	66.3

	5.5	Lact.	115	9	681			374	292	+3	112	
	5.6	SBM	349	11	2016	l-cystine	inactive	798	1095	+22	327	59.7
	5.7	SBM	343	11	2016	l-cystine	inactive	794	1135	+15	328	57.7
	5.7	SBM	343	11	2016	l-cystine	inactive	669	1055	+51	292	61.7
	5.8	SBM	286	11	1720	l-cystine	active	700	963	+10	276	60.4
	5.8	SBM	286	11	1720	l-cystine	active	688	871	+28	258	65.8
	5.8	SBM	286	11	1720	l-cystine	active	680	875	+28	258	65.5
	5.8	Lact.	109	8	681			455	334	−19	128	
	5.7	Lact.	111	8	681			342	330	+2	109	
	5.8	SBM	331	9	1970	none	inactive	840	1265	−23	354	50.1
	5.8	SBM	331	9	1970	none	inactive	879	1135	−8	339	56.7
	5.7	SBM	337	9	1970	none	inactive	931	1202	−29	366	53.3
	5.7	SBM	305	9	1793	none	active	852	866	+13	292	67.4
	5.8	SBM	300	9	1793	none	active	858	847	+15	285	68.5
	5.8	SBM	300	9	1793	none	active	911	808	+13	287	70.7
	5.6	Lact.	113	9	681			378	257	−8	105	
	5.7	Lact.	111	9	681			321	285	−13	98	
75	6.8	Lact.	105	9	780			555	289	−9	114	
	6.7	SBM	334	9	2301	l-cystine	inactive	988	1361	−7	341	53.5
	6.7	SBM	334	9	2301	l-cystine	inactive	1014	1319	−5	339	55.3
	6.4	SBM	350	9	2301	l-cystine	inactive	1025	1293	−3	353	56.4
	6.2	SBM	310	10	1983	l-cystine	active	981	1041	−6	316	62.1
	6.2	SBM	310	10	1983	l-cystine	active	1088	943	−8	318	67.1
	6.1	SBM	315	10	1983	l-cystine	active	937	937	+18	297	67.4
	6.1	Lact.	117	10	780			576	284	−13	130	
	6.2	Lact.	115	10	780			462	270	+8	107	
	6.3	SBM	334	10	2165	dl-methion.	inactive	804	1118	+39	295	61.8
	6.2	SBM	340	10	2165	dl-methion.	inactive	918	1132	+19	321	61.1
	6.1	SBM	345	10	2165	dl-methion.	inactive	932	1087	+24	321	63.2

TABLE 1—*Concluded*

Dog. no.	Weight	Nitrogen source	Nitrogen Intake: test protein	Nitrogen Intake: basal diet	Total N-intake	Amino acid supplement	Caroid	N-excretion: urinary	N-excretion: fecal	Nitrogen balance	Calculated N-intake at equilibrium	Digestibility
	kg.		mg./kg./day		mg./day			mg./day		mg./kg./day	mg./kg./day	%
	6.1	SBM	270	10	1711	dl-methion.	active	937	828	−9	279	68.6
	6.0	SBM	275	10	1711	dl-methion.	active	892	802	+3	272	70.1
	5.9	SBM	279	11	1711	dl-methion.	active	818	797	+16	263	70.4
	5.9	Lact.	121	10	780			540	309	−12	133	
	6.0	Lact.	119	10	780			443	300	+6	113	
77	9.5	Lact.	109	9	1130			747	460	−8	117	
	9.5	SBM	340	9	3313	l-cystine	inactive	1185	2220	−10	350	45.8
	9.5	SBM	340	9	3313	l-cystine	inactive	1078	2244	−1	341	45.1
	9.7	SBM	333	9	3313	l-cystine	inactive	1090	2142	+8	325	48.2
	9.7	SBM	284	9	2850	l-cystine	active	1142	1419	+30	254	65.1
	9.7	SBM	284	9	2850	l-cystine	active	1071	1645	+14	270	57.2
	9.8	SBM	282	9	2850	l-cystine	active	997	1430	+43	239	64.7
	9.9	Lact.	105	9	1130			582	400	+15	90	
	10.1	Lact.	103	9	1130			550	424	+15	90	
	10.3	SBM	330	8	3483	dl-methion.	inactive	1089	2157	+23	307	50.3
	10.3	SBM	330	8	3483	dl-methion.	inactive	1009	2055	+41	289	53.2
	10.3	SBM	330	8	3483	dl-methion.	inactive	994	2099	+38	292	51.9
	10.4	SBM	249	9	2679	dl-methion.	active	888	1361	+41	208	65.1
	10.4	SBM	249	9	2679	dl-methion.	active	924	—	—	—	—
	10.4	SBM	249	9	2679	dl-methion.	active	886	1314	+46	203	66.8
	10.5	Lact.	99	9	1130			571	440	+11	88	
	10.7	Lact.	97	9	1130			531	402	+18	79	

* These latter contained either active or inactive papain and, in some instances, were supplemented with sulfur amino acids as indicated.

Montgomery *et al.*[13] reported that ingested trypsin exerted its activity in eight depancreatized dogs and noted, without giving their data, that under the same approach papain was also effective upon oral administration. They also observed positive effects in all their dogs. These two studies are, therefore, in essential agreement, since it was observed, in each instance, as was the case in the rat feeding trials referred to above,[1, 2] that *in vivo* enzyme digestive activity can be demonstrated after oral ingestion of proteolytic enzymes.

Effect of Papain on the Soy Bean Nitrogen Intake Required for Equilibrium. The effect of papain on the amount of soy bean nitrogen required by the dogs for equilibrium (nitrogen intake = nitrogen output) was calculated in the following way:

nitrogen intake at equilibrium = nitrogen intake − nitrogen balance.

TABLE 2

THE INFLUENCE OF PAPAIN ON THE DIGESTIBILITY OF SOY BEAN MEAL PROTEIN FED TO DOGS

Dog no.	*Endogenous nitrogen excretion*	*Metabolic nitrogen excretion*	*Amount of protein digested, means ± s.e.*		*P*
			inactive papain	*active papain*	
	mg./day	*mg./day*	%	%	
55	529	203	52.9 ± 1.5	60.5 ± 1.0	<0.001
62	774	359	44.4 ± 0.2	54.0 ± 2.0	0.006
64	742	295	60.7 ± 1.4	65.1 ± 2.6	0.15
66	689	350	47.8 ± 1.8	62.0 ± 2.0	<0.001
70	528	247	59.2 ± 1.7	60.3 ± 1.7	0.2
72	416	282	57.2 ± 1.3	65.5 ± 1.1	<0.001
73	558	272	66.5 ± 1.0	69.5 ± 2.3	0.12
75	515	290	58.6 ± 1.5	67.6 ± 1.3	0.001
77	596	425	49.1 ± 1.3	63.8 ± 1.6	0.001
Average			54.7 ± 1.1	63.1 ± 0.7	<0.001

Since amino acid supplementation had a pronounced effect upon the equilibrium nitrogen value, the data of TABLE 1 are averaged in TABLE 3 by groups in accordance with the supplement employed.

Considering all the results of TABLE 3, it may be seen that invariably less soy bean nitrogen was required to maintain the dogs in nitrogen equilibrium when their diet was supplied with active papain than when inactive papain was fed. In only five out of the fifteen individual dietary periods compared were the differences not far beyond the probable limits of spontaneous variability.

Considering the data by groups, it may be seen that eight per cent less nitrogen was required to maintain the dogs in equilibrium when active papain was fed along with the unsupplemented diets. Fed along with the cystine supplemented diets, active papain effected a 12 per cent reduction in the amount of nitrogen required to maintain the dogs in equilibrium. An even greater effect on nitrogen equilibrium was noted when the methionine supplemented diets were fed. In the latter instance, an 18 per cent re-

duction in the amount of nitrogen required to maintain the dogs in equilibrium resulted.

Thus, as in the case of the comparisons based upon the extent of protein digestion, it may be seen that papain activity may be demonstrated convincingly *in vivo*. The markedly improved responses of most of the dogs due to the active papain are the more significant because the increases were superimposed on normal digestive activities.

Effect of Cystine or Methionine Supplements on the Biological Value of Soy Bean Protein. While these tests had for their primary interest the

TABLE 3
CALCULATED SOY BEAN NITROGEN INTAKES REQUIRED FOR EQUILIBRIUM IN DOGS FED INACTIVE OR ACTIVE PAPAIN IN THEIR DIETS

Dog no.	*Amino acid supplement*	*Soy bean nitrogen intake at equilibrium, means ± s.e.*		*P*
		inactive papain	*active papain*	
		mg. N/kg./day		
55	none	318 ± 3	309 ± 10	0.4
62	none	326 ± 5	308 ± 7	0.9
66	none	363 ± 3	329 ± 5	<0.001
72	none	353 ± 8	288 ± 2	0.002
Average		340 ± 2	312 ± 1	<0.001
55	l-cystine	303 ± 4	267 ± 6	<0.001
64	l-cystine	261 ± 8	242 ± 16	0.3
66	l-cystine	354 ± 12	290 ± 10	0.003
70	l-cystine	293 ± 3	275 ± 3	0.006
72	l-cystine	324 ± 7	274 ± 5	<0.001
73	l-cystine	270 ± 8	257 ± 13	0.2
75	l-cystine	344 ± 4	310 ± 7	0.013
77	l-cystine	339 ± 7	254 ± 9	0.001
Average		310 ± 3	271 ± 2	<0.001
73	dl-methionine	245 ± 11	218 ± 3	0.13
75	dl-methionine	312 ± 9	271 ± 5	0.014
77	dl-methionine	296 ± 6	206 ± 3	0.001
Average		289 ± 7	237 ± 8	<0.001

effect of orally ingested papain on protein digestion, the data permit calculations of the biological value of soy bean protein by Mitchell's formula:[12]

$$\text{Biological Value} = \frac{\text{Food N} - (\text{Fecal N} - \text{Metabolic N}) - (\text{Urinary N} - \text{Endogenous N})}{\text{Food N} - (\text{Fecal N} - \text{Metabolic N})} \times 100$$

When thus calculated, the biological value is apparently uninfluenced by papain (P = 0.1 or more). Consequently, the data for active and inactive papain were combined to give both the biological value of the soy bean protein when fed unsupplemented, or when fed supplemented with cystine or methionine.

The biological value of the unsupplemented soy bean protein, as determined in dogs equilibrated at minimal nitrogen intakes, was 54.0 (s.e. ±

1.2, 4 dogs, 38 weeks of feeding). That for cystine supplemented soy bean meal was 69.1 (s.e. ± 1.1, 8 dogs, 70 weeks of feeding). That for the methionine supplemented protein was 76.3 (s.e. ± 1.6, 3 dogs, 15 weeks of feeding). These values were significantly different from each other ($P = <0.001$).

The value for raw soy bean protein found here is in good agreement with that of 57, given by Block and Mitchell in their extensive review.[14]

Lactalbumin Nitrogen Intake at Equilibrium. It is of interest to compare the amount of lactalbumin nitrogen found necessary to maintain the dogs in equilibrium with findings of other investigators. Melnick and Cowgill[15] observed that dogs require 175 to 200 mg. of lactalbumin nitrogen per kg. per day to maintain them in nitrogen equilibrium. More recently, Kade *et al.*[9] reported the requirement to be 90 mg. of lactalbumin nitrogen per kg. per day. These may be compared with the value derived from these studies (9 dogs, 44 weeks of observations) of 104 (s.d. ± 14) mg. lactalbumin nitrogen per kg. per day. The value is lower than the one submitted by Melnick and Cowgill for the probable reason that these investigators did not equilibrate their dogs at minimal nitrogen intakes before placing them on test. Kade *et al.* indicated that their procedure of calculating the minimal nitrogen intakes, based upon the calculated optimum weight of the dogs, rather than the actual weights, as used by Risser[7] and as used here, yielded values somewhat lower than would have been obtained if the actual weights of the dogs had been used. As stated above, we have preferred to calculate the caloric needs of the dogs on the basis of the Cowgill-Drabkin formula, but have expressed the results in terms of the actual weights of the animals, rather than upon calculated ones.

Summary

The *in vivo* activity of papain added to the diet has been studied in nine normal dogs maintained near or at nitrogen equilibrium after equilibration at minimal protein intakes. The protein source material in these studies was unheated, defatted soy bean meal.

On the basis of the results obtained, the following comparisons may be made:

Papain added to the diet significantly increased the digestion of soy bean protein, even above the normal digestive capacity of these animals. The difference in three of the nine dogs was barely significant statistically but was beyond the range of probable spontaneous variation in the remaining six. The overall average was an increase in the protein digested from 54.6 per cent to 63.1 per cent, or an improvement in digestive efficiency of 15.5 per cent above the normal.

Active papain added to the diet significantly reduced the amount of soy bean protein required to maintain the dogs in nitrogen equilibrium. The results were the same, whether or not the protein was supplemented with cystine or methionine.

It was noted that the biological value of soy bean protein was not affected by papain added to the diet. When pooled, the data indicated that the soy

bean protein had a biological value of 54.0 (s.e. ± 1.2). Supplementing the soy bean protein with one per cent cystine increased the biological value to 69.1 (s.e. ± 1.1). One per cent methionine increased it to 76.3 (s.e. ± 1.6).

An incidental observation of the study indicated that 104 (s.d. ± 14) mg. of lactalbumin nitrogen per kilogram per day were required to keep the dogs in nitrogen equilibrium.

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